



**RAPID MICROCHEMICAL METHODS FOR  
BLOOD AND C.S F EXAMINATIONS**



# Rapid Microchemical Methods for Blood and C S F Examinations

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*With a foreword by F SILBERSTEIN, M D*



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## Foreword

By the end of 1937 only two years after it had been published, the first edition of Rappaport's 'Mikrochemie des Blutes' was out of print, and a second enlarged manuscript was almost ready for the press. The annexation of Austria by Nazi Germany brought the scientific life in Vienna to a standstill. In those circumstances it appeared uninteresting to put a new German edition into circulation even if it had been possible. The author was forced to leave Vienna and to build up a new working place both for the daily routine and research work. Rappaport has always been teeming with brilliant ideas, and technical skill and ingenuity are characteristic of him. He mastered all obstacles, and in spite of enormous difficulties his laboratories became a scientific centre the importance of which has been recognised far beyond the Middle East.

There spurred by the exacting tasks that wartime conditions imposed upon him he devised numerous new methods and amended and improved others. The shortage of laboratory equipment, the impossibility of obtaining many of the chemicals which until then had been thought indispensable, was for him just another impulse to find new and often better ways of microbiological or biochemical analysis.

I feel honoured for having been asked by my dear friend, Dr Fritz Rappaport, to write a preface to his book. Our research unit of the once famous Institute for General and Experimental Pathology of the University of Vienna has been scattered to the four winds. The spiritual union based on sincere friendship on common work, and above all upon common love of science and humanity, still holds good, steered and strengthened by unhappy experience. For many years we have been separated from one another and working in different parts of the globe. Rappaport, however, has kept us informed of his work. No sooner had he devised a new method than he communicated it to us and we made use of it to the benefit of our hospitals and laboratories. Today we can state that the new Rappaport methods have proved their value wherever put to the test.

Many up-to-date books on biochemical and microbiological methods are at hand. In spite of this I am convinced that the monograph "Rapid Microchemical Methods for Blood and C.S.F. Examina

tions," is supplying a great want. It contains many excellent methods which are hardly to be found elsewhere as they have either not yet been published or are hidden from wider publicity in journals of a very restricted circulation. The new book brings to the fore a stimulating account of these new ways of microbiological determination which lay open an approach to various most important problems which hitherto could not be tackled. To quote one example. Our knowledge of the protein metabolism is admittedly very poor, and in many respects not sufficiently supported by analytical data. That is to a great extent due to the fact that also the recent methods of Rest-N determination are still much too cumbersome to be used for mass analyses in clinics or at the bedside. Rappaport's micro-estimation of non protein N (without digestion and distillation) has rendered the Rest-N determination into being a really simple and reliable bedside test which does not tax our time and skill more than a routine blood-count or a blood sugar determination. Let us keep in mind that important progress in medicine and biology has mostly been preceded by the discovery of new methods of assessment and investigation, e.g., after Mering and Minkowski had shown that diabetes could be established in dogs at will by removing the pancreas, time and again the effect of various pancreatic extracts has been tried on test animals. As long as no suitable micromethod of blood sugar determination was available only the general reaction of the test animals could be observed and that of course doomed all the efforts to failure. The convulsions with ensuing death of the animals which followed the injection of potent pancreatic extracts, were interpreted as originating in the "primary toxicity" of the extracts as their real cause, hypoglycemia, had at that time not been established for lack of a suitable micromethod. It was Bang's pioneer work and the contribution of other laboratory workers that paved the way for Banting and Best. In such circumstances it would be difficult to overestimate the importance of technical improvement for scientific discoveries.

I trust this book will be widely circulated not only among biochemists and clinical pathologists but especially also among medical people interested in research. The reliability of the methods described in it has been tested assiduously and repeatedly. Rappaport has made a special point of giving a detailed and minutely exact

description of all technical procedures. Thus it should be an easy task to assure the correctness of the determination, even when the tests are not performed by highly trained specialists. I hope this book will meet with the success it well deserves and that it will not only add to the efficiency of our laboratories but help to bring more light in some of the dark corners of our physiological and pathological conception. *Quod felix, faustum fortunatumque sit!*

F SILBERSTEIN

*Former Professor of General and Experimental Pathology of the University of Vienna, former Laboratory Director of the S Canning Childs Hospital and Research Institute Vienna, and former Laboratory Director of the F Pearson Research Foundation at the West London Hospital London*





# Preface

This book contains microchemical methods for the analysis of blood and spinal fluid. These methods have been tested and have been proven satisfactory

Some of the methods represent original work. They have been worked out in part in my former laboratory, the Institut für all gemeine und experimentelle Pathologie in Vienna (Professor F Silberstein director now resident in London) and in part in the Laboratory of the Beilinson Hospital of the Kupath Cholim in Petach Tiqua

Drawing on the experience of an earlier book I shall attempt to proceed along much the same lines established in that work and to accomplish the following objects

- 1 to simplify the laboratory procedures
- 2 to decrease the amount of material needed for analysis,
- 3 to shorten the working time,
- 4 to increase the precision of the results
- 5 to stabilize the solutions wherever possible,
- 6 to work with simple apparatus. For this reason, expensive equipment such as photometers and photoelectric colorimeters are only mentioned

Many methods have been adapted to subtropical climates, others have been changed and made less expensive these changes arising in large part from wartime experiences with scarcity of materials.

I am happy to express gratitude to my teacher, Professor Silberstein, who has guided this work with his advice and counsel

My longtime assistant, Mrs Friedl Eichhorn added much to the success of the work with her diligence and tireless help

I also wish to express my thanks to Dr V T Meier Dr H Hellor and to all the physicians of the Beilinson Hospital in Petach Tiqua for their generous help. I am grateful to Dr N Eichhorn for the careful revision and correlation of the manuscript and to Dr A Fried for the sketching and execution of the drawings

F RAPPAPORT M D

*Petach Tiqua, Israel*  
*January, 1949*



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## Chapter I

# Determination of the General and Physical Qualities of the Blood

### DETERMINATION OF SPECIFIC GRAVITY<sup>1</sup>

*Principle of the method* A short 0.15 ml precision pipet which has been calibrated with distilled water is used in the procedure. The tip and the markings should be very narrow so that absolutely nothing of the contents can flow out or evaporate. In order to keep the pipets in a horizontal position on the balance scale, a small support made of aluminum wire is used, the weight of which together with the weight of the pipet, has to be determined with each use (fig. 1). The pipet is weighed exactly to the fourth decimal point (0.1 mg), then it is filled with serum or blood and again weighed.

#### *Determination of Specific Gravity of Serum*

The pipet is dried to constant weight held with a pair of forceps, and the serum is pipetted to the mark through rubber tubing. The outside of the pipet is wiped off carefully; it is left upon the balance scale for 10 minutes and weighed to the fourth decimal point (0.1 mg).

#### *Calculation*

$$\text{Spec. grav} = \frac{\text{weight of liquid at } t^{\circ}}{\text{weight of an equal volume of water at } t^{\circ}} = \frac{W_1 - W_0}{W_2 - W_0}$$

$W_1$  = weight of pipet and serum

$W_0$  = weight of the empty pipet

$W_2$  = weight of pipet and water

This formula applies only when the water and the test liquid are weighed at the same temperature and under the same conditions.

#### *Example*

weight of pipet and water at 20° C	4.0225
weight of empty pipet, at 20° C	3.8724
difference	0.1501

is equal to the volume of the pipet in ml at 20°C

weight of pipet and serum at 20 °C	4.0277
weight of pipet at 20 °C	3.8724
weight of serum.	0.1553

$$\text{spec. grav} = \frac{0.1553}{0.1501} = 1.0414$$

In order to save repeated weighing of water at different temperatures it is sufficient to multiply the volume of the pipet indicated on the certificate by the weight of 1 ml of water at the required temperature (table 1) and divide the weight of the unknown sample by this figure

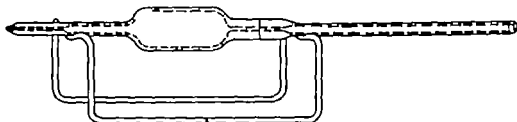


FIG 1 Micropipet for the determination of the specific gravity according to Pregl

### Example

Determination at 25°C

Volume of pipet = 0.15 ml

Weight of 1 ml. of water at 25°C = 0.99618 G (table 1)

$$\text{spec. grav} = \frac{\text{weight of 0.15 ml. of serum at 25°C}}{0.15 \times 0.99618}$$

If no calibrated pipet is available, the volume of a red count pipet or a white count pipet may be determined. The pipet is weighed empty, then with water. (Since the weight of 1 ml of water at any given temperature is known, the volume of the pipet can be calculated as follows

$$\text{spec. grav} = \frac{G}{V} \quad V = \frac{G}{\text{spec. grav}}$$

TABLE 1 —Weight Calibration Table

Temperature (°C)	Weight of 1000 ml. of water under normal laboratory conditions (grams)
15	997.93
16	997.80
17	997.65
18	997.51
19	997.34
20	997.18
21	997.00
22	996.80
23	996.61
24	996.39
25	996.18
26	995.94
27	995.70
28	995.45
29	995.19
30	994.92
31	994.64
32	994.35
33	994.06
34	993.75
35	993.45

I Kolthoff and E Sandell Textbook of quantitative inorganic analysis  
New York The Macmillan Co 1943 p 527

### Example

The amount of water in the pipet at 24°C weighs 0.2764 G At 24°C 1 ml of water weighs 0.99639 G The volume of the pipet is therefore

$$V = \frac{0.2764}{0.99639} \quad V = 0.2774 \text{ ml.}$$

The protein content of serum or plasma may be calculated from the specific gravity as follows\*

$$(a) \text{ plasma g per cent protein in plasma} = (\text{spec grav of plasma} - 1.0089) \times 340$$

According to the formula of Week quoted in John Scudder \*Shock  
p 9

(b) serum g per cent protein in serum = (spec grav of serum minus 1.0073)  $\times$  347.9

### Example

spec grav of serum = 1.0260

1.0260

1.0073

0.0187       $0.0187 \times 347.9 = 6.51$  g per cent protein in serum.

The determination of serum (or plasma) proteins may also be done by the copper sulfate method of Van Slyke and co-workers (see p. 9)

### *Determination of Specific Gravity of Whole Blood and Plasma (Native)*

A trace of Liquoid (Roche) is placed on a small watch-glass in order to prevent coagulation, and a drop of blood drawn from the finger tip or from the ear lobe is added to this. The liquid is then pipetted into a small pipet as described above.

When a series of determinations is to be made it is recommended to dissolve 100 mg. of Liquoid in 100 ml. of distilled water. Of this solution 0.04 ml. are placed upon a small watchglass. After the water has been evaporated in the incubator, the glasses are ready to be used for reception of the blood.

The second weighing (pipet plus blood) should be done very quickly. *Calculation*, as described for serum.

Normal density of blood 1050 to 1060

Normal density of serum 1025 to 1030

During great loss of water the density of blood may rise to 1070, that of serum to 1050.

### DETERMINATION OF THE WATER CONTENT

*Principle of the method* A small amount of blood is absorbed on filter paper and dried to constant weight at 100°C. The weight loss after drying represents the water content of the sample.

#### *Procedure*

A small piece of filter paper (Whatman) is placed in a small weighing bottle and dried for one hour in the drying oven at 100°C, the lid being left open. Then the bottle and lid are placed separately into

a desiccator After cooling the bottle is closed and weighed on the analytic balance Blood is drawn and absorbed very quickly by the filter paper The paper should be held with a pair of forceps and care should be taken that the blood is distributed evenly The paper is placed immediately into the weighing bottle, the lid is replaced, and it is weighed Then the lid is removed and together with the bottle it is dried for one hour at 100 C, allowed to cool in the desiccator as described above, and reweighed. After this weighing it is again left to dry in the oven for one hour and again weighed after proper cooling. This procedure is repeated until the weight is constant.

### Calculation

The weight of the sample used can be calculated from the weight of the weighing bottle plus filter paper before absorption of the blood and after absorption of the blood on the paper The water content equals the weight of the bottle with wet paper minus weight of bottle with paper after drying From these three data the water content and the dry residue of the blood can be determined and expressed in per cent. Water content of sample equals  $X$  minus  $Y$   $X$  being the weight of blood before drying and  $Y$  the weight of blood after drying

$$\text{Water content in per cent} = \frac{100 \times (X - Y)}{X}$$

$$\text{Dry residue in per cent} = \frac{Y \times 100}{X}$$

### Example

weight of weighing bottle + paper	5.9235 g
weight of weighing bottle + paper + blood (wet)	6.1037 g
weight of blood	0.1802 g
weight of bottle + paper + blood after drying	5.9417 g
weight of bottle + paper	5.9235 g
dry residue	0.0182 g

water content of sample expressed in per cent

$$\frac{(0.1802 - 0.0182) \times 100}{0.1802} = 89.00 \text{ per cent}$$



dry residue in per cent

$$\frac{0.0182 \times 100}{0.1802} = 10.10 \text{ per cent}$$

Normal water content of serum 92 per cent

Normal water content of whole blood 80 per cent



FIG. 2 Copper sulfate bottle with eye dropper for the determination of specific gravity

#### DETERMINATION OF THE SPECIFIC GRAVITY OF WHOLE BLOOD AND SERUM WITH THE COPPER SULFATE METHOD<sup>1</sup>

*Principle of the method* A drop of blood or serum of a certain specific gravity which is allowed to fall through a copper sulfate solution from a capillary pipet will either rise, float, or fall to the bottom according to the specific gravity of the copper sulfate solution. The specific gravity of the copper sulfate solution which just keeps the sample afloat, corresponds to the specific gravity of the blood or serum.

*Apparatus* Twenty bottles (100 ml) with screw top for the determination of the specific gravity of serum and 40 bottles (100 ml) with screw top for the determination of the specific gravity of whole blood. Eyedropper (fig. 2)

### *Solutions*

The copper sulfate solutions of the various specific gravities are prepared from a stock solution of the specific gravity 1.100 according to table 2a.

The stock solution is best prepared according to K. Steinitz.\* Approximately 185 g  $\text{CuSO}_4$  are dissolved in water in a one-liter volumetric flask and the flask is made up to the mark with distilled water. Care must be taken that the salt is "analytical reagent" and that it gives a clear solution in water. Five ml. of this solution should use up 31.96 ml. of N/10 neutral thiosulfate solution. If more thiosulfate is required the copper sulfate solution has to be diluted accordingly. If less thiosulfate is used more copper sulfate has to be added. The titration must be done as follows: to 5 ml. of the copper sulfate solution is added 1 to 2 g. potassium iodide. The flask is then covered with a watch glass, and is titrated after one minute with thiosulfate to a faint yellow. Then starch solution is added and the titration is continued until the solution is colorless. (A faint pink color will remain). From this stock solution the bottles are filled and each one is carefully labeled. Since each bottle can only be used for 100 drops it is advisable to mark the bottles each time after use. It is further recommended that they be mounted upon a round rotating stand to facilitate the work.

### *Procedure*

A small amount of whole blood or serum is taken up with an eye-dropper which is equipped with a rubber bulb and one drop is allowed to fall through the liquid from a height of 1 cm. It is best to start within the normal range, which is around 1.025 for serum and about 1.052 for whole blood. The next solution is selected according to the behavior of the drop, i.e., if the drop falls through the copper sulfate solution a bottle containing a solution of higher specific gravity is next used. If the drop rises a bottle containing a solution of lower specific gravity is chosen until the correct solution is found which will keep the drop of whole blood or serum afloat over a period of a few seconds. The surface of the drop will coagulate when in

Personal communication from Dr. K. Steinitz, Director of Laboratory Haifa Hadassah Hospital.

TABLE 2a—*All of stock copper sulfate solution of gravity 1.000 to be diluted to 100 ml to prepare standard solution of gravity G to within 0.0001*

G	100†	G	100
1.008	7.33	1.038	87
09	8.32	39	88
10	9.31	40	89
11	10.30	41	90
12	11.29	42	91
13	12.28	43	92
14	13.27	44	93
15	14.26	45	94
		46	95
16	15.25	47	96
17	16.24	48	97
18	17.23	49	98
19	18.22	50	99
20	19.21	51	100
		52	101
21	20.20	53	102
22	21.19	54	103
23	22.17	55	104
24	23.16	56	105
25	24.15	57	106
		58	107
26	25.14	59	108
27	26.13	60	109
28	27.12	61	110
29	28.11	62	111
30	29.10	63	112
		64	113
31	30.09	65	114
32	31.08	66	115
33	32.07	67	116
34	33.06	68	117
35	34.05	69	118
36	35.04	70	119
37	36.03	71	120
		72	121
		73	122
		74	123
		75	124

\* G = specific gravity of standard solution

† 100 = ml of 1.000 stock solution diluted to 100 ml

TABLE 2b

Sp Gr	Gm. per cent
1 017	4 00
17 5	4 18
18	4 38
18 5	4 55
19	4 73
19 5	4 91
20	5 09
20 5	5 27
21	5 46
21 5	5 64
22	5 82
22 5	6 00
23	6 18
23 5	6 37
24	6 55
24 5	6 73
25	6 91
25 5	7 09
1 26	7 28
26 5	7 46
27	7 64
27 5	7 82
28	8 00
28 5	8 19
29	8 37
29 5	8 55
30	8 73
30 5	8 91
31	9 10
31 5	9 28
32	9 46

contact with the copper sulfate solution and therefore will appear cloudy

#### Calculation

The calculation of the total proteins from the specific gravity is made according to the formula of H Hoch and J Marrack<sup>2</sup>

$$\text{Protein} = 364 \times (\text{specific gravity minus } 1.000)$$

The values obtained by this formula agree well with those obtained by the Kjeldahl method

If this method is used for the determination of serum proteins, it is advisable to mark on the label not only the specific gravity of the solution, but also the corresponding protein content (table 2b)

For the purposes of the Blood Donor Service and for general health service, a single solution, of a specific gravity of 1.032, corresponding to 12.3 g of hemoglobin permits the acceptance or rejection of a donor without obtaining the exact amount of hemoglobin in that donor's blood. In other words, if a drop of donor's blood sinks in the solution the donor is acceptable, if the drop rises or remains suspended the individual's hemoglobin is less than acceptable standard, that is less than 12.3 Gm.<sup>4</sup>

#### DETERMINATION OF THE SEDIMENTATION RATE OF THE RED BLOOD CELLS<sup>5</sup>

*Principle of the Method* According to Reichel, the sedimentation rate is independent of the height of the blood column if the depth of the sedimentation does not exceed one third of the entire column. It is also independent of the volume of the tube provided the diameter of the latter is not smaller than 1.4 mm. With a narrower tube the capillary action would interfere perceptibly with the determination. The sedimentation rate also depends on the external temperature, being accelerated by heat and retarded by cold.<sup>6, 7</sup> In order to exclude this source of error—which in tropical countries shows its effect especially in the summer, while in temperate zones it is more conspicuous in the winter—the following apparatus was constructed

#### *Apparatus*

A wide-necked thermos flask (fig. 3a) fitted with an inner rack to hold the sedimentation pipets. The stand is built to hold six sedimentation pipets, springs keeping it at a given height. At the bottom of the stand six small test tubes with side apertures (2–3 mm above the lower end) are fixed by means of small springs. The sedimentation pipets are placed into these tubes when a determination is carried out.

Glass tubes (fig. 3b) with a diameter of 1.45–1.6 mm. approximately 21 cm long the lower part without graduation and cut flat. The ungraduated part corresponds to one-fourth the graduated part i.e., from 30–40 mm. The upper graduated section is divided into

centimeters, showing millimeter graduation as well. The length of the graduated part is from 120-160 mm. Over the top a soft and transparent tubing of para rubber is fitted containing a movable glass bead.

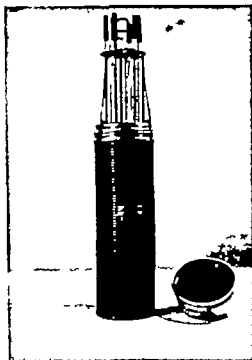
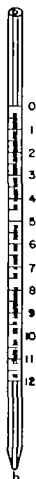


FIG 3a. Apparatus for the determination of the sedimentation rate

FIG 3b Sedimentation rate tube



### Reagents

- (1) 3.8 per cent neutral sodium citrate solution (tribasic)
- (2) glycerin to moisten the tubing

### Procedure

The pipets should be rinsed with distilled water and dried with warm air only (no acetone or alcohol should be used). Before blood is drawn from the finger tip the entire pipet should be rinsed with

citrate solution. Then the citrate should be sucked up to the ungraduated part. This citrate is blown out into a small test tube. The blood is then allowed to flow (by capillary action) into the pipet—which should lie in a position as horizontal as possible—up to the mark 0. Then the blood is allowed to flow into the citrate test tube, and mixed well, care being taken to avoid formation of bubbles. The sedimentation test may be set up immediately, or, at the latest, in the course of the next five hours. If the sedimentation is set up immediately, the pipet may be used again without being cleaned beforehand; otherwise it may be used only after thorough cleaning, drying and renewed moistening with citrate solution.

For setting up the reaction, the tube is moistened with a small drop of glycerin at its lower end. Almost the entire length of rubber tubing (about 4 cm.) is slipped over the pipet. The glass bead pressed aside, the blood citrate mixture should be sucked up to mark 1. By drawing the rubber tubing upward the blood column is raised up to mark 0. (If necessary, correct by shifting the bead inside the tubing.) Now the pipet is inserted into the stand which is put into the thermos flask, previously filled with water at 20°C. Readings are taken after thirty, sixty, and 120 minutes at a slow sedimentation and after thirty and sixty minutes only, if the sedimentation is fast.

The sedimentation rate is increased in all infectious and degenerative processes, it is decreased in polycythemia.

Normal values: males 2-5 mm after 1 hour, females 3-8 mm after 1 hour.

Evaluation of the 1 hour reading according to Krause & Wichmann<sup>8</sup>

Males	Females
Less than 2 mm. retarded	Less than 3 mm.
2-5 mm. normal	3-8 mm. (except during menstrual period and pregnancy)
6-10 mm. border line	9-12 mm. menstrual period and pregnancy
11-20 mm. slight acceleration	26-35 mm. pregnancy
21-30 mm. moderate acceleration	36-60 mm.
31-60 mm. strong acceleration	more than 60 mm.
More than 60 mm. very strong acceleration	

(See under heading HEMATOCRIT for 'sedimentation' p. 21, sedimentation rate dependent upon red cell volume)

#### DETERMINATION OF THE COAGULATION BAND ACCORDING TO WELTMANN<sup>9-11</sup>

*Principle of the Method* Serum which has been diluted 50 times loses

addition of minimal amounts of an electrolyte (0.1 per cent to 0.01 per cent of a  $\text{CaCl}_2$  solution). The amount of electrolyte necessary is called the electrolytic threshold. The electrolytic threshold differs in various diseases. (1) Original method according to Weltmann

### Reagents

A stock solution of exactly 5 per cent  $\text{CaCl}_2$  (i.e., approximately 10 per cent  $\text{CaCl}_2 \times 0.5\text{H}_2\text{O}$ ) is required. Since  $\text{CaCl}_2$  is highly hygroscopic, the salt must be dried in a desiccator before it is weighed. The solution is made up by dissolving 99.14 G  $\text{CaCl}_2$  cryst. reagent in 1000 ml of sterile redistilled water. This solution will keep indefinitely.

Calcium chloride will contain water even after drying in the desiccator. Therefore, the following procedure for making up the stock solution is recommended. Starting with commercial  $\text{CaCl}_2$  which need not be dry, a concentrated solution is made up, the chlorine content of which is determined with  $\text{AgNO}_3$  (see titration of chlorides, p. 68). One ml. of a Weltmann stock solution should correspond to 9.05 ml of N/10  $\text{AgNO}_3$  solution. If more  $\text{AgNO}_3$  is required, the solution must be diluted according to the equation. If less  $\text{AgNO}_3$  is used up, more  $\text{CaCl}_2$  must be added and the titration repeated.

*Example* One ml. of  $\text{CaCl}_2$  solution prepared as described above requires 11.2 ml of N/10  $\text{AgNO}_3$ . The solution is too concentrated and must be diluted according to the following formula:

$$\frac{9.05}{11.2} = \frac{X}{1000}$$

$$X = \frac{9050}{112} = 808$$

Accordingly, 808 ml. of  $\text{CaCl}_2$  solution must be made up to 1000 ml. with distilled water.

A series of  $\text{CaCl}_2$  dilutions is prepared (0.1 per cent–0.01 per cent) by pipetting into 100 ml. volumetric flasks 0.1, 0.2, 0.3, 0.35, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml. of the stock solution and filling to the mark with redistilled water. These dilutions are kept in eleven glass stoppered bottles which should be numbered I–VII, VIIb–X, beginning with the highest concentration.

Calcium chloride may be replaced by calcium lactate or calcium gluconate both of which have the advantage of being obtainable as



dry powder so that the weighing of an exact amount is not difficult. 1.39 G. of calcium lactate or 2.019 G. of calcium gluconate per 100 ml. solution corresponds to a 1 to 10 dilution of the Weltmann stock solution. The Weltmann solutions I to X are prepared by measuring into each 100 ml. volumetric flask amounts from 10 ml. to 1 ml. respectively of the stock solution and filling to the mark with redistilled water.

Calcium lactate and calcium gluconate readily deteriorate as a result of the action of molds and must therefore be stabilized by adding strips of filter paper, containing mercuric iodide, which are prepared in the following manner. Powdered  $HgI_2$  is mixed with liquefied paraffin and this mixture is poured over filter paper. The  $HgI_2$  is fixed on the filter paper and serves as an excellent bactericide (see also determination of blood sugar).

The test is performed by pipetting 5 ml. of each concentration, starting with concentration X into a clean dry test tube. Then 0.1 ml. of cell free, nonhemolyzed serum is added to each tube. The tubes are shaken well and placed in a boiling water bath for fifteen minutes. After that time they are read. The reaction is called positive up to and including the tube in which a definite precipitate can still be seen. A milky appearance alone is negative.

The reaction may also be carried out by making up a 0.1 per cent  $CaCl_2$  solution from the solution by diluting it 100 times and setting up the following series of tubes.

	I	II	III	IV	V	VI	VII	VIIb	VIII	IX	X
$CaCl_2$ (ml.)	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.75	1.5	1.0	0.5
$H_2O$ (ml.)	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.25	3.5	4.0	4.5
result conc.	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.035	0.03	0.02	0.01

In order to facilitate pipetting it is recommended that the tubes be placed in the rack according to the following pattern so that the indicated amount of  $CaCl_2$  solution may be pipetted onto two opposite tubes. They are then filled with water to 5 ml.

If the exact end point is to be determined, it is recommended that a tube marked VIIb be inserted between tube VII and tube VIII and

that to this tube be filled with 1.75 ml of  $\text{CaCl}_2$  solution and 3.25 ml of water

	II	III	IV	V		
	(4.5)	(4.0)	(3.5)	(3.0)		
I	0.5	1.0	1.5	2.0	VI	
(5.0)					(2.5)	(0.1 per cent $\text{CaCl}_2$ , water.)
0.0	(0.5)	(1.0)	(1.5)	(2.0)	2.5	
	4.5	4.0	3.5	3.0		
	X	IX	VIII	VII		

The rest of the procedure (adding the serum heating and reading) is performed as described above

### *Micromethod<sup>13</sup>*

#### *Reagents*

$\text{CaCl}_2$  cryst. reagent is dried in a vacuum desiccator and 100.91 G are dissolved in water in a 1000 ml. volumetric flask. From this stock solution the working solution is prepared by diluting 1.0 ml with water to 100 ml in a volumetric flask. The working solution may also be prepared from the stock solution described in (1) by diluting 1.08 ml to 100 ml with distilled water

#### *Procedure*

Eleven Wassermann tubes are placed in a metal rack (65 mm. long, 16 mm. diameter). The tubes are filled with  $\text{CaCl}_2$  solution and with redistilled water according to the following scheme

	I	II	III	IV	V	VI	VII	VIII	VIIIb	VIII	IX	X
$\text{CaCl}_2$ (ml)	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.35	0.3	0.2	0.1	
$\text{H}_2\text{O}$ (ml)	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.65	0.7	0.8	0.9	

In order to facilitate pipetting the following scheme may be used (for explanation see *macro* method)

	II	III	IV	V		
	(0.9)	(0.8)	(0.7)	(0.6)		
I	0.1	0.2	0.3	0.4	VI	
(1.0)					(0.5)	( $\text{CaCl}_2$ )
0.0	(0.1)	(0.2)	(0.3)	(0.4)	0.5	$\text{H}_2\text{O}$
	0.9	0.8	0.7	0.6		
	X	IX	VIII	VII		

In making up these dilutions it is advantageous to use serologic pipets whose tips have been slightly bent (fig 4) With these pipets

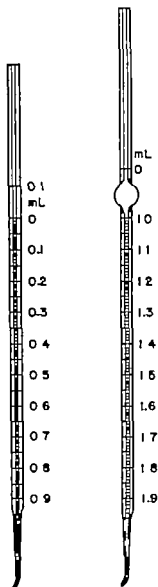


FIG 4 Improved serologic pipet permitting transfer of small amounts of liquid without loss

small amounts of liquid may be measured out with great accuracy. Very little adherence of the liquid to the glass wall will occur.

Three tenths of a milliliter of the serum to be tested is diluted with 1.1 ml of redistilled water and 0.1 ml of this serum dilution is

pipetted with the above described pipet into each tube. After a fifteen minute immersion in a boiling waterbath they are read.

In the micromethod the stronger calcium chloride solution is used to balance the weakening of the electrolyte which occurs in diluting serum, the final concentrations in both methods must be identical. In the *macro* determination one part of serum corresponds to 50 parts of the electrolyte solution (total volume 5.1 ml. hence,  $\frac{5.1}{50} = 1.02$ ).

According to the formula

$$\frac{1.02}{1.1} = \frac{99.14}{X}$$

$$X = \frac{99.14 \times 1.1}{1.02} = 106.91$$

To make up the stock solution 106.91 G. of crystallized  $\text{CaCl}_2$  must be dissolved in 1 liter of redistilled water. In this stock solution 1 ml. corresponds to exactly 9.76 ml. of N/10  $\text{AgNO}_3$  solution. If the *macro* stock solution is to be used the following calculation shows that 1.08 ml. of this solution must be made up to 100 ml. with water.

$$\frac{1.02}{1.10} = \frac{100}{X}$$

$$X = \frac{110}{1.02} = 1.078 \quad \text{ie.,} \quad 1.08$$

As mentioned above in the *macro*-method, 0.1 ml. of serum reacts with 5 ml. of the electrolyte solution; this proportion must be maintained when using the *macro* method.

According to the formula

$$\frac{5.1}{1.1} = \frac{0.1}{X}$$

$$X = 0.0215$$

In the *micro*-method 0.0215 ml. of serum react. For accurate measurement of these small amounts, 0.3 ml. of serum is diluted with 1.1 ml. of water, according to the following formula

$$0.0215 \times 14 = 0.3$$

*Micromethod with Macroreagent**Reagents* see original method (solution I)*Procedure*

Two hundredths of a milliliter of serum is delivered on the bottom of eleven small tubes. This is done with an 0.2 ml pipet bent at its end and graduated in 1/1000 parts of ml. Then a solution of 0.1 per cent calcium chloride and water are added according to the following scheme

Nr	I	II	III	IV	V	VI	VII	VIIb	VIII	IX	X
CaCl <sub>2</sub> (ml)	1 0	0 0	0 8	0 7	0 6	0 5	0 4	0 35	0 3	0 2	0 1
redistilled water (ml)	0 0	0 1	0 2	0 3	0 4	0 5	0 6	0 65	0 7	0 8	0 9

To facilitate the work, the following pattern may be used

	II (0 9) 0 1	III (0 8) 0 2	IV (0 7) 0 3	V (0 6) 0 4		VI (0 5) 0 5	(CaCl <sub>2</sub> ) H <sub>2</sub> O
I (1 0) 0 0	(0 1) 0 9 X	(0 2) 0 8 IX	(0 3) 0 7 VIII	(0 4) 0 6 VII			

For routine determinations or in cases where very little serum is available it suffices to fill only six tubes, filling one with the concentration Nr V. After fifteen minutes boiling the reaction may be read. If flocculation occurs in this tube the series is continued to the right according to the above scheme. If the tube remains clear, the series is continued to the left.

The normal values for the coagulation band fall between tubes VI and VII. A coagulation over VII must be considered prolonged, (shift to the right) and one less than VI shortened (shift to the left).

A prolonged band can be found in all fibroid processes, mainly in cirrhosis of the liver and in all hemolytic processes. Shortening of the band occurs in exudative and inflammatory processes, in cases of kidney damage, in necrotic processes, independent of the cause of the

necrosis (carcinoma lymphogranuloma, tissue degeneration, vascular obstruction, coronary infarct)

### TAKATA ARA REACTION

A colloidal suspension of mercuric chloride in alkaline medium ( $\text{Na}_2\text{CO}_3$ ) remains unchanged in the presence of normal serum proteins. In certain pathologic conditions changes occur which lead to flocculation of serum proteins in a number of dilutions.

#### *Original methods*

##### (A) *Macromethod*

###### *Reagents*

(1) 10 per cent sodium carbonate solution, (dissolve 10 G of anhydr  $\text{Na}_2\text{CO}_3$  in 100 ml of water)

(2) 0.25 per cent sublimate solution

(3) 0.9 per cent sodium chloride solution

Reagents (1) and (2) are not stable they will keep in the icebox for two to four weeks. It is important to use only the purest grade of  $\text{Na}_2\text{CO}_3$ , e.g., the product by May & Baker

###### *Procedure*

A series of eight regular test tubes is filled with 1 ml of NaCl solution (3) beginning with the second tube. Into each of the first two tubes is placed 1 ml of serum. A dilution series is now made by transferring after mixing well, 1 ml from the second tube into the third and from there into the fourth tube, etc. The last milliliter from tube 8 is discarded. To each tube is now added 0.25 ml of carbonate solution (1) and 0.3 ml of the sublimate solution (2). After twenty four hours at room temperature the tubes are read.

The reaction is positive when at least two or three tubes show flocculation, regardless of the dilution.

(B) *Micro-method*. The reaction may also be performed as follows

###### *Reagents*

(1) 11 G NaCl and 2.5 G anhydr  $\text{Na}_2\text{CO}_3$  are dissolved in water and made up to 100 ml

(2) 0.125 G  $\text{HgCl}_2$  is dissolved in water and made up to 100 ml

###### *Procedure*

Eight regular test tubes are filled with 0.25 ml of reagent (1) and serial dilutions are made by adding 0.25 ml of serum to the first tube mixing

well and transferring 0.25 ml of the mixture into the second tube, etc. Then each tube receives 0.15 ml of reagent (2). Further treatment and reading is performed as described for the *macro* method.

The use of the serologic pipet according to Rappaport (fig. 4) is recommended. This pipet with a bent tip permits transferring of very small amounts of liquid without loss.

This reaction is positive in compensated cirrhoses of the liver, in serious catarrhal icterus, occasionally also in certain bone and kidney diseases.

### *Takata Ara reaction (one glass method) for spinal fluid<sup>13</sup>*

Into a small narrow test tube 1 ml of spinal fluid is placed and to this is added one drop (0.05 ml) of a 10 per cent solution of sodium carbonate and 0.3 ml of a freshly prepared mixture of equal parts of 0.5 per cent sublimate solution and 0.02 per cent Fuchsin solution. Then the tubes are shaken vigorously. In specific luetic meningitis a blue-violet precipitate is formed below a colorless supernatant. In nonluetic meningitis a spontaneous pink coloring appears without a precipitate. The possible readings may be summarized as follows:

Normal	blue-violet no flocculation
Pathologic	(1) late luetic type* blue-violet flocculation, clear supernatant
	(2) meningitis type pink color no flocculation

In late luetic processes a quantitative reading can be done by reading after fifteen minutes, thirty minutes, and twenty-four hours.

++++ after 15-30 minutes clear supernatant, flocculation below

+++ after 24 hours blue-violet precipitate with clear supernatant

++ after 24 hours clear supernatant only half size

+ after 24 hours clear supernatant layer only 1-2 mm wide

Serologic microtests see: An improvement of the serological Kahn reaction in the spinal fluid. F. Rappaport and D. Rappaport. *J. Lab. and Clin. Med.* 28 Nov. 11 1943. Rapid test for the serodiagnosis of syphilis. F. Rappaport and F. Eichhorn. *Lancet* April 3 1943 p. 426. *Lancet* Nov. 4 1944 p. 599.

HEMATOCRIT<sup>14</sup>

The relationship of red cell volume to the total volume of whole blood is disturbed in various diseases. The hematocrit indicates the percentage of packed red blood cells in whole blood.

*Reagents*

Liquoid (Roche)

*Apparatus* U-shaped capillary glass tube.

*Preparation* This tube can easily be made from a capillary of constant diameter. Melting point tubes or damaged 0.1 ml. pipets are suitable. With the aid of paper graduated in millimeters a mark is made on the glass at the 100 mm. point. Then the tube is carefully heated in the center over a small flame. As soon as the glass begins to melt it is bent to form a U with two unequal sides (5 mm. difference), the shorter side bearing the mark (fig. 5).

*Procedure*

Rubber tubing is attached to the short arm of the U. On a small watch glass containing a spot of Liquoid powder to prevent clotting a few drops of blood from the finger or the ear lobe are mixed quickly. The blood is sucked up through the long side of the U with the aid of the rubber tubing until it exactly reaches the mark. Then the column of blood measuring 100 mm. is sucked up further until equal heights are reached in both arms of the U. The tube is centrifuged for fifteen minutes at high speed. After centrifuging it is placed on millimeter paper and the height of the plasma columns are read.

*Calculation*

$$100 - a - b = \text{hematocrit value}$$

*a* and *b* represent the heights of the plasma columns.

In a different method a straight tube is used instead of the U shaped tube, one end of which is sealed off with wax before centrifuging. In order to obtain exact values with this method a mark affixed at 80 and 100 mm. is recommended.

*Procedure*

After mixing with Liquoid the blood is sucked up to the mark. Then the column is sucked a little higher and the tube is sealed off.



with wax. After centrifuging for fifteen minutes at high speed, the erythrocyte column is measured

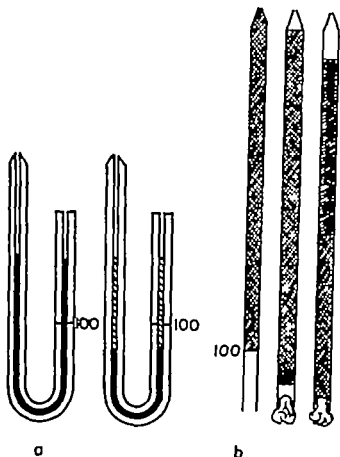


FIG 5 HEMATOCRIT TUBES

### Calculation

Erythrocyte column height (100 mm mark) = hematocrit value.

Erythrocyte column height (80 mm mark)  $\times \frac{4}{3}$  = hematocrit value

An increased hematocrit value can be found in all diseases where dehydration has taken place, i.e., burns, shock, dysentery, cholera, polycythemia

A low hematocrit is found in all kinds of anemia, after hypertonic NaCl infusions, in hemorrhage and in sepsis.

### *Correction of the Sedimentation Rate for Anemia<sup>15 16</sup>*

The complex factors, both physical and chemical, which govern the rate of sedimentation of the red cells have not yet been clearly defined.

Nevertheless the sedimentation test has a practical value because experience has shown that persons who have an increased sedimentation rate cannot be regarded as normal and that they are usually suffering from some organic disease which causes tissue destruction and toxemia. The disease is commonly an infection, a malignant growth, or extensive simple ulceration. The sedimentation rate is not in

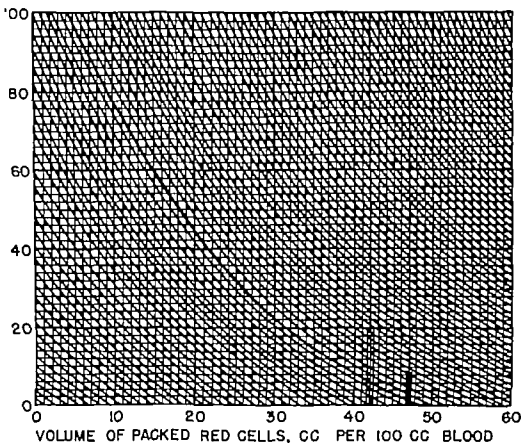


FIG 6 CORRECTION CHART FOR SEDIMENTATION RATE DETERMINATION

creased in uncomplicated functional disorders such as pernicious anemia (Britton 1936) but when to such a functional disorder are added other factors—e.g., sepsis, neoplasma or tuberculosis—then the sedimentation rate becomes increased.

An anemic blood sediments rapidly by reason of the anemia itself. Hence the observed sedimentation rate is not the true sedimentation rate. It is desirable therefore, to apply a proper correction to differentiate anemias with a functional basis from those secondary to

organic disease. Furthermore the progress of any disease process could be more accurately followed by serial sedimentation rates if it were possible to distinguish between the effects due to variations in the activity of the primary disease and those due to the associated secondary anemia.

With the aid of the cell volume (hematocrit) it is possible to find the corrected values for the sedimentation rate, using the chart (fig. 6) <sup>17</sup>

#### DETERMINATION OF THE FRAGILITY OF RED BLOOD CELLS

*Principle of the method* A series of test tubes are filled with sodium chloride solutions of various concentrations (0.7 per cent–0.3 per cent) and measured amounts of freshly drawn blood are introduced in the tubes. After standing for two hours, the concentrations which lead to beginning or complete hemolysis are observed.

#### Reagents

1 per cent NaCl solution accurately weighed out. To 10 gm of NaCl is added 1000 ml of redistilled water.

#### Procedure

The following pattern is used in filling twenty-one test tubes (diameter 10 mm height 90 mm) with 1 per cent NaCl solution and making up to 1 ml with distilled water.

	(0 52)	(0 54)	(0 56)	(0 58)	(0 60)	(0 62)	(0 64)	(0 66)	(0 68)	(0 70)
(0 50)	0 48	0 46	0 44	0 42	0 40	0 38	0 36	0 34	0 32	0 30
0 50	(0 48)	(0 46)	(0 44)	(0 42)	(0 40)	(0 38)	(0 36)	(0 34)	(0 32)	(0 30)
	0 52	0 54	0 56	0 58	0 60	0 62	0 64	0 66	0 68	0 70
	(1 per cent NaCl)					distilled water				

In order to facilitate pipetting it is advantageous to set up the tubes according to the above illustrated pattern and to pipet the indicated amounts of NaCl solution out of a one ml pipet into two opposite tubes. Presently the water is added in the reverse order to bring the volume to 1 ml. The amounts of NaCl used correspond to the NaCl concentrations in each tube.

With a capillary pipet about 0.025 ml of blood is transferred directly from the finger into the test tubes. The tubes are shaken in

mediately to avoid clotting. Several pipets should be kept for use. The tubes are shaken and allowed to stand for two hours then they are centrifuged. The beginning of hemolysis (minimum resistance) is recognized by the first faint redness of the supernatant fluid complete hemolysis (maximal resistance) manifests itself by the absence of red cells on the bottom of the tubes.

The reading is facilitated by placing the tubes over a mirror (preferably the convex mirror of a microscope). The presence of undamaged erythrocytes is then easily visible. When an angle centrifuge is used the undamaged erythrocytes are seen as a red point on the wall of the centrifuge tube.

The hemolysis of normal blood starts between 0.42 and 0.44 per cent NaCl concentration (minimal resistance). It ends between 0.36 and 0.32 per cent NaCl concentration (maximal resistance).

A decreased minimal resistance is found in hyperchromic anemia, in erythroblastic anemia and in polycythemia. A decreased maximal resistance is found in hemolytic jaundice as well as in aplastic anemia. An increased maximal resistance is found in hyperchromic anemia and after splenectomy. In the latter case hemolysis may be entirely absent.

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## Chapter II

# Blood Gas Analysis according to D D van Slyke (Volumetric and Manometric)

*Principle of the method* : The blood gases are liberated in an evacuated chamber. In this chamber carbon dioxide, oxygen, nitrogen, and carbon monoxide (when present) are liberated together and then determined separately by absorption with specific reagents. The measurement of the gases is done either volumetrically or manometrically.

### VOLUMETRIC DETERMINATION OF CARBON DIOXIDE IN PLASMA (ALKALI RESERVE)

*Apparatus* The apparatus (fig 7) consists mainly of a 50 ml chamber with a 2-way stopcock sealed on to both ends. The upper part of the chamber (pipet) is calibrated for 1 ml in divisions of 0.01 or 0.02 ml. The adjoining wider part of the pipet carries marks at 1.25 ml, 1.50 ml, 2.0 ml, and 2.5 ml. The 50 ml mark is placed at the bottom end of the pipet.

The upper stopcock connects the chamber with a capillary outlet and with a graduated funnel or cup used for filling. The lower stopcock forms the connection with the two side-arms of a U-shaped tube (reservoir), the arms being of different width. The wider arm holds the liquid after the liberation of the carbon dioxide, the narrower allows the passage of mercury after release of the vacuum at the end of each analysis. The bottom of the U tube is connected with heavy rubber tubing to a leveling bulb which is equipped with a leveling tube at the side for easier reading. The entire apparatus must be made of strong glass and should be attached to a heavy iron stand with a clamp lined with rubber tubing. The leveling bulb is held in place by two hooks, one of which is attached at the height of the first 2-way stopcock (position 1) and the other at the height of the second stopcock (position 2). Before each analysis the chamber is washed 2 or 3 times with water acidified with sulfuric or lactic acid. Sometimes the entire apparatus must be cleaned. To this effect it is left

over night in cleaning solution (sulfuric acid-dichromate solution). Care must be taken that the acid does not reach the rubber tubing.

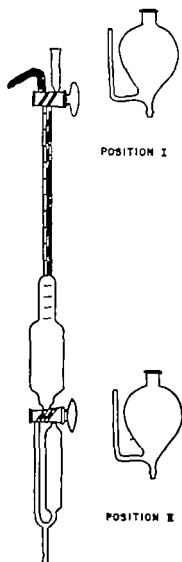


FIG. 7 Volumetric blood gas apparatus according to van Slyke

After that the apparatus is washed with water. When not in use the chamber and funnel should remain filled with water.

Heavy walled vacuum tubing should be used. This tubing is boiled in vaseline in vacuum in order to prevent contamination by

**Hg sulfide formation** As a stopcock grease the following mixture is recommended

**Vaseline-rubber lubricant \*** One part of pure, nonvulcanized, raw rubber gum is cut into small pieces and dissolved in 4 parts of vaseline and 1 part of paraffin. The mixture is heated in an oven at 110°C for about two days until the rubber becomes a uniform mass. Subsequently the mixture is heated with a micro-burner for a half-hour at 150°-160°C and strained through cotton gauze. This lubricant is applied to the core of the stopcock after it has been covered with a thin layer of vaseline. At low room temperature more pure vaseline and less vaseline-rubber lubricant is used.

The stopcock must be scrupulously cleaned before it is lubricated. In order to keep the bore of the stopcock grease-free, a minimal amount of it has to be applied in a ring about each end of the stopcock and the core of the cock turned in its casing. A well lubricated cock should appear transparent and turn with perfect ease.

Another good greasing agent is prepared by melting together 2 parts of lanoline and 1 part of white wax.

Before each determination the stopcocks must be tested for vacuum. An ordinary glass cock will not hold a vacuum unless the bore of the cock is filled with mercury. In using the vacuum apparatus, both the volumetric and the manometric one, the curved capillary outlet at the left of the cock of the chamber is kept continually filled with mercury, likewise the bore leading through the cock to this capillary. The other bore leading to the cup above the chamber, must be filled with mercury each time before the chamber is evacuated.

The following procedure is used to test whether the apparatus is gas-tight. The entire apparatus is filled with mercury by lifting the leveling bulb, then the upper stopcock (position 1) is closed and sealed with mercury. Now the bulb is lowered and by keeping the lower stopcock open, the mercury is allowed to drop to the 50 mark. If there is a leak air will be sucked in and when the bulb is now lifted the mercury will not reach the upper stopcock due to the presence of air. If the apparatus is air tight the mercury will hit the upper stopcock with a noticeable noise when the leveling bulb is raised. If this is not the case the procedure must be repeated and gas expelled through the funnel until no more air is present. Repeated evacuation

This lubricant can be obtained from Eimer & Amend, New York City.

is necessary when new rubber tubing is used, or if air has been allowed to enter the tubing. If repeated evacuation fails to secure vacuum, the stopcocks must be removed and regreased after careful cleaning.

A better procedure to test for vacuum is the following. About 0.4–0.5 ml of air is introduced into the apparatus, the upper stopcock is closed, sealed with mercury and the volume of air is read by leveling

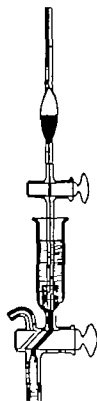


FIG. 8 Delivery of blood sample into chamber of apparatus from rubber tipped pipet.

the mercury in the bulb with the mercury in the apparatus. Then the bulb is lowered to bring the mercury to the 50 mark. the lower stopcock is closed. the apparatus is removed from the stand and shaken by hand 10–15 times. The apparatus is then attached to the stand and the mercury is allowed to enter the chamber by opening the lower stopcock and lifting the bulb. Now the gas volume is read. If the apparatus was air tight, the reading will be the same as before.

*Pipets.* If 1 ml of plasma is used the Ostwald pipet (fig. 8) is



recommended, which allows the measurement of blood between 2 marks, one mark being above the bulb, the other mark below the bulb of the pipet, both marks are above the stopcock of the pipet. The pipet is tipped with a soft rubber ring, made of a piece of rubber tubing 1 cm. in length, 1 mm inner diameter and 2 mm thickness. This rubber tipped pipet is used to transfer the sample directly into the chamber of the apparatus. If no Ostwald pipets are available ordinary pipets with 2 marks may be used

### *Reagents*

(1) Approximately N/1 lactic acid prepared by diluting 1 part of concentrated lactic acid (specific gravity 1.2) with 10 parts of water. Acid as well as water must be free from carbon dioxide. To remove possible traces of  $\text{CO}_2$ , air is blown through the dilute acid until the  $\text{CO}_2$  content is reduced to 0.03 volume per cent, which is in equilibrium with the  $\text{CO}_2$ -tension of the atmosphere. To 100 ml. of dilute lactic acid 1 ml. of caprylic alcohol (octyl alcohol) is added.

(2) 1 per cent ammonia,  $\text{CO}_2$ -free. The 1 per cent ammonia solution containing a few drops of methylorange as indicator is shaken with barium hydroxide to remove  $\text{CO}_2$ . The barium carbonate is filtered off and the excess barium hydroxide is removed from the filtrate by precipitation with ammonium sulfate.

(3) 10 per cent sulfuric acid

### *Preparation of the blood for analysis*

(1) *Bleeding* From a nontourniqueted vein, 5-10 ml. of blood are drawn and delivered carefully into a tube containing a mixture of equal parts of sodium or lithium oxalate and sodium fluoride and a 2 cm. layer of mineral oil. The blood must not come in contact with air during the delivery. It is mixed carefully with a thin glass rod, and as soon as possible centrifuged and the plasma separated from the blood. The oil is used to prevent loss of  $\text{CO}_2$  when the blood is stirred. This loss may cause a transfer of  $\text{Cl}^-$  ions from red blood cells to plasma and so significantly diminish the  $\text{CO}_2$  combining power.

(2) *Saturation of plasma with alveolar carbon dioxide* After centrifugation of the blood sample 3 ml. of the supernatant clear plasma is pipetted into a clean 250-300 ml. separatory funnel. The outlet of

the funnel is connected with rubber tubing to a wash bottle, filled to 3/4th of its volume with glass beads (for the absorption of water vapors—(fig 9). In order to obtain a large surface the funnel is held in a horizontal position and expired air is blown as quickly and as completely as possible through the wash bottle 12 times. After each breath the funnel is closed and the plasma spread out along the walls of the funnel by turning and tilting. In this manner the complete saturation of the plasma with  $\text{CO}_2$  (approximately 40 mm Hg) is accomplished. Then the plasma is transferred carefully to a test tube containing mineral oil.

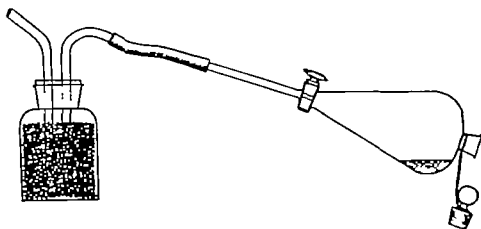


FIG 9 Separatory funnel containing plasma and serum arranged for filling with alveolar air

### Procedure <sup>1</sup>

#### (A) *Determination using Ostwald pipets*

The entire apparatus including the capillary outside of the stopcock is filled with mercury by lifting the leveling bulb. The upper stopcock is closed and the bulb is lowered a little. The mercury column in the reading capillary must remain intact. The dilute lactic acid with which the plasma will react is placed in the filling cup of the apparatus. With the Ostwald pipet 1 ml of plasma is taken up the outside of the pipet is carefully wiped off and the rubber ring is placed on the tip. The pipet is then placed against the bottom of the cup and an airtight connection obtained. The plasma is now allowed to flow into the chamber by opening the upper stopcock of the

apparatus and the stopcock of the pipet. As soon as the plasma has reached the lower mark of the pipet the flow of the sample is interrupted by closing the stopcock of the pipet, the pipet is removed and the upper stopcock of the apparatus is closed. These operations must be carried out rapidly and simultaneously. Then the acid is introduced into the chamber to reach the 2.5 ml mark and the cock sealed with mercury. By lowering the bulb (position 2) the mercury is forced through the chamber to the 50 ml mark, the lower stopcock is closed, the apparatus is removed from the stand and the liquid is thoroughly mixed by tilting the pipet 15 times. Then the apparatus is reattached to the iron stand and by slowly opening the lower stopcock the aqueous layer except for a very small portion is allowed to enter the wider arm of the U-shaped tube (reservoir). A small quantity is left behind to prevent the extracted gasses from entering the reservoir from the pipet, otherwise the analysis must be discarded. Now the bulb is raised and the stopcock is turned in such a way as to make connection with the second smaller reservoir (narrow arm of the U tube). By careful lifting of the bulb the mercury is now directed into the chamber. The mercury will fill up the chamber with the exception of the space taken up by the liberated  $\text{CO}_2$  and the rest of the liquid. The mercury in the bulb is brought to the same level as in the capillary tube of the pipet and the amount of liberated  $\text{CO}_2$  is read off at the meniscus of the liquid. Then the lower stopcock is turned and mercury is allowed to take the place of the aqueous content of the chamber. By opening the upper stopcock and lifting the bulb the liquid is removed from the apparatus through the upper capillary tube. Now the apparatus is washed with water and is ready for the next determination.

*Calculation.* The alkali reserve for temperatures between  $15^\circ\text{C}$  and  $30^\circ\text{C}$  is calculated according to the following formula.

$$X = \frac{B}{760} \times (100.8 - 0.27 \times t)(V - 0.136 + 0.002 \times t)$$

X represents the ml of  $\text{CO}_2$  (reduced to  $0^\circ\text{C}$  and 760 mm Hg) with which 1 ml of plasma can combine as bicarbonate after shaking with alveolar air (5.5 per cent  $\text{CO}_2$  = about 40 mm Hg) at  $20^\circ\text{C}$ .

B = barometric pressure

t = temperature

V =  $\text{CO}_2$  volume

The calculation is simplified by the table of van Slyke and Cullen.<sup>1</sup>

The alkali reserve is calculated from  $\frac{V \times B}{760}$  The ratio  $\frac{B}{760}$  is found in table 3a and V can be found in table 3b

*Example* At 18°C and 744 mm Hg barometric pressure 0.02 ml of CO<sub>2</sub> are liberated from 1 ml of plasma.  $\frac{B}{760}$  from table 3a gives

TABLE 3a — *Barometric Reduction Table*

Barometric pressure read off	Barometer 760	Barometric pressure read off	Barometer 760
732	0.061	756	0.095
734	0.066	758	0.097
736	0.067	760	1.000
738	0.071	762	1.003
740	0.074	764	1.006
742	0.076	766	1.008
744	0.079	768	1.011
746	0.081	770	1.013
748	0.084	772	1.016
750	0.087	774	1.018
752	0.089	776	1.021
754	0.092	778	1.024

0.079 for B 744. Consequently 0.02 has to be multiplied by the factor 0.079 = 0.00158 = 0.01. From the table can be read

under 0.01 for 15° C

48.7 ml CO<sub>2</sub>

under 0.01 for 20° C

49.0 ml CO<sub>2</sub>

The difference for 5° is 0.3 ml for 1 it is 0.06 ml CO<sub>2</sub>. Thus 0.18 ml (0.06 × 3) are to be added to the value for 15°. The alkali reserve i.e. the amount of ml CO<sub>2</sub> with which 100 ml of plasma can combine under the above described conditions, is

$$48.7 + 0.18 = 48.88 \text{ ml of CO}_2$$

(B) *Determination performed with regular volumetric pipets*

At the beginning of the determination the leveling bulb is brought into position 1 and the entire apparatus including both capillaries is filled with mercury. The filling cup is filled with several drops of 1 per cent ammonia (2). (If a red color appears due to acid present

TABLE 3b—Table for the calculation of CO<sub>2</sub> combining power of plasma

Amount of gas read off B x 760	cm. <sup>3</sup> CO <sub>2</sub> reduced to 0° 760 mm. bound as bicarbonate by 100 cm. <sup>3</sup> of plasma				Amount of gas read off B x 760	cm. <sup>3</sup> CO <sub>2</sub> reduced to 0° 760 mm., bound as bicarbonate by 100 cm. <sup>3</sup> of plasma			
	15°	20°	25°	30°		15°	20°	25°	30°
0 20	0 1	0 0	10 7	11 8	0 00	47 7	48 1	48 5	48 6
1	10 1	10 0	11 7	12 0	1	48 7	49 0	49 4	49 5
2	11 0	11 8	12 0	13 5	2	49 7	50 0	50 4	50 4
3	12 0	12 8	13 0	14 3	3	50 7	51 0	51 3	51 4
4	13 0	13 7	14 5	15 2	4	51 6	51 9	52 2	52 3
5	13 0	14 7	15 5	16 1	5	52 6	52 8	53 2	53 2
6	14 0	15 7	16 4	17 0	6	53 6	53 8	54 1	54 1
7	15 0	16 0	17 4	18 0	7	54 5	54 8	55 1	55 1
8	16 8	17 0	18 3	18 0	8	55 5	55 7	56 0	56 0
9	17 8	18 5	19 2	19 8	9	56 5	56 7	57 0	56 9
0 30	18 8	19 5	20 2	20 8	0 70	57 4	57 6	57 9	57 9
1	19 7	20 4	21 1	21 7	1	58 4	58 6	58 9	58 8
2	20 7	21 4	22 1	22 0	2	59 4	59 5	59 8	59 7
3	21 7	22 3	23 0	23 5	3	60 3	60 5	60 7	60 6
4	22 6	23 3	24 0	24 5	4	61 3	61 4	61 7	61 6
5	23 6	24 2	24 9	25 4	5	62 3	62 4	62 6	62 5
6	24 6	25 2	25 8	26 3	6	63 2	63 3	63 6	63 4
7	25 5	26 2	26 8	27 3	7	64 2	64 3	64 5	64 3
8	26 5	27 1	27 7	28 2	8	65 2	65 3	65 5	65 3
9	27 5	28 1	28 7	29 1	9	66 1	66 2	66 4	66 3
0 40	28 4	29 0	29 6	30 0	0 80	67 1	67 2	67 3	67 1
1	29 4	30 0	30 5	31 0	1	68 1	68 1	68 3	68 0
2	30 3	30 9	31 5	31 9	2	69 0	69 1	69 2	69 0
3	31 3	31 9	32 4	32 8	3	70 0	70 0	70 2	69 9
4	32 3	32 8	33 4	33 8	4	71 0	71 0	71 0	70 8
5	33 2	33 8	34 3	34 7	5	71 9	72 0	72 1	71 8
6	34 2	34 7	35 3	35 6	6	72 9	72 9	73 0	72 7
7	35 2	35 7	36 2	36 5	7	73 9	73 9	74 0	73 6
8	36 1	36 6	37 2	37 4	8	74 8	74 8	74 9	74 5
9	37 1	37 6	38 1	38 4	9	75 8	75 8	75 8	75 5
0 50	38 1	38 5	39 0	39 3	0 90	76 8	76 7	76 8	76 4
1	39 1	39 5	40 0	40 3	1	77 8	77 7	77 7	77 3
2	40 0	40 4	40 9	41 2	2	78 7	78 6	78 7	78 2
3	41 0	41 4	41 9	42 1	3	79 7	79 6	79 6	79 2
4	42 0	42 4	42 8	43 0	4	80 7	80 5	80 6	80 1
5	42 9	43 3	43 8	43 9	5	81 6	81 5	81 5	81 0
6	43 9	44 3	44 7	44 9	6	82 6	82 5	82 4	82 0
7	44 9	45 3	45 7	45 8	7	83 6	83 4	83 4	82 9
8	45 8	46 2	46 6	46 7	8	84 5	84 4	84 3	83 8
9	46 8	47 1	47 5	47 6	9	85 5	85 3	85 2	84 8
0 60	47 7	48 1	48 5	48 6	0 100	86 5	86 2	86 2	85 7

the ammonia must be removed the funnel washed with water, dried with a roll of filter paper and refilled with ammonia) The tip of the pipet is placed carefully under the liquid level and 1 ml. of plasma, prepared as described above is allowed to run out slowly By opening the upper stopcock and lowering the bulb the plasma is let into the chamber The funnel is washed twice with 0.5 ml. of boiled water 1 drop of caprylic alcohol and enough 10 per cent sulfuric acid are added to bring the mercury to the 2.5 mark. The rest of the procedure and the calculation are carried out as before.

Protective glasses must always be worn when working with the volumetric apparatus (danger of breakage vacuum)

#### MANOMETRIC SEMIMICRO-DETERMINATION OF $\text{CO}_2$ IN PLASMA (ALKALI RESERVE)

The use of the manometric apparatus<sup>1</sup> is desirable if greater accuracy is necessary, if gases other than  $\text{CO}_2$  are also to be determined or if only small amounts of blood (0.2 ml.) are available The results are of highest accuracy since either volume or pressure of the extracted gases may be varied according to the purpose aimed at for each determination

##### *Principle of the method*

The blood gases are liberated by the addition of suitable reagents and by evacuation. The liberated gases are adjusted to a known volume The pressure ( $p_1$ ) of this volume of gas in the chamber is read on a mercury manometer Then the gases are either expelled from the chamber or absorbed by suitable reagents and the pressure ( $p_2$ ) is read at the same volume as before absorption The partial pressure  $P$  of the gas at a given volume equals  $p_1 - p_2$  mm. Hg. From this difference the gas volume at  $0^\circ\text{C}$  and 760 mm. Hg can be calculated. Corrections for vapor pressure and capillary attraction can be omitted since they are the same for both readings, provided that the temperature remains constant this is generally the case during the short time interval of the determination The barometric pressure will also remain constant during this period.

##### *Description of the apparatus*

The extraction chamber (fig. 10) of 50 ml. volume is calibrated at 0.5 ml., 2 ml. and 50 ml. The chamber is additionally subdivided

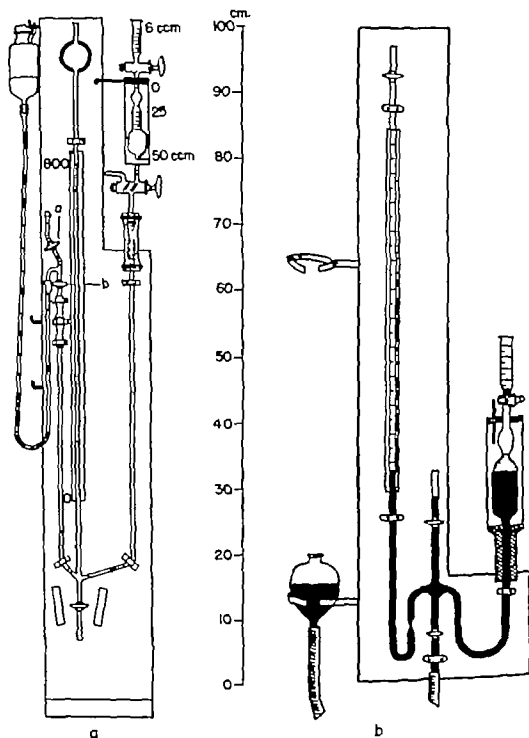


FIG 10 Apparatus for the manometric blood gas determination (van Slyke)  
a With open manometer b With closed manometer

between 2 ml and 4 ml to measure the extraction liquid. A stop-cock with a 1.2-1.3 mm bore connects the chamber with the measuring cup which holds approximately 7 ml. and through which the reagents are introduced into the chamber. The cup is graduated in 0.5 ml divisions.

The extraction chamber is held in a water jacket serving as a constant temperature bath. The temperature is read on a thermometer inserted in the water. There is an airtight connection between the lower part of the chamber and a long vertical glass tubing.

(The airtight connection is obtained as follows. The end of the chamber is pushed through the hole of a rubber stopper. A piece of vacuum tubing, previously boiled in vaseline in vacuum, is slipped over the open end of the chamber. A piece of longer and wider tubing is slipped over the rubber stopper. A rubber stopper of equal size is put on the long vertical glass tubing, which is then connected to the chamber through the vacuum tubing so as to leave a distance of about 5 mm between the glass parts. The glass ends should not touch each other otherwise the chamber may break when shaking. Now the lower end of the wide rubber tubing is moistened with rubber solution, slipped over the lower rubber stopper and fastened with wire. The outer rubber tubing is filled with mercury with the aid of a small funnel inserted between the upper end of the rubber tubing and the stopper. The connection thus becomes airtight. The funnel is removed, the upper end of the jacket is closed, sealed with rubber and tied with wire.)

The inner diameter of the glass tubing is 4 mm, its length 800 mm. It is bent at right angles and is in a free connection with a leveling bulb and a mercury manometer. Its upper end may be either open or closed (fig 10a). In the *open* manometer a pear shaped end prevents loss of mercury by spilling when the bulb is lifted above the extraction chamber. Oscillations of mercury between the extraction chamber and the manometer are minimized by the narrowing of its lower end. The zero point is located about 80 cm below the 50 ml mark of the chamber. The manometer tube is subdivided in distances of 1 mm. Each 10th division is marked by a heavy line. In order to avoid parallax errors a circular marking is recommended. The reading is facilitated by placing a piece of frosted glass behind the tube.

Instead of the open manometer whose zero point is at knee height



a closed manometer can be used (fig 10b) The closed manometer consists of a tube of 4 mm diameter which bears no opening, but is closed with a stopcock The calibration starts at the height of the middle of the chamber and has divisions up to 600 mm At the beginning of the determination the air is expelled from the manometer by filling it with mercury and the stopcock is closed Thus no atmospheric pressure is exerted on the mercury in the manometer and consequently all the readings rise by 760 mm. This apparatus is much shorter than the one with open manometer and can easily be transported But there are certain disadvantages resulting from the danger of the reagents penetrating into the closed manometer The ensuing water vapor pressure may lead to faulty pressure measurements. In order to cancel this error 3-4 drops of concentrated sulfuric acid or trimethylene-glycol or dimethylene-glycol are admitted through the manometer stopcock They are allowed to flow down the tube for about 10 cm and are again ejected through the stopcock by raising the mercury level The remaining acid or glycol absorbs any possible water vapors.

The bulb of the leveling apparatus has a volume of 100 ml and is connected through 1 meter of vacuum tubing (boiled in vasoline in vacuum) to a Y-shaped joint with 2 stopcocks. The stopcock (a) at the upper end of the Y joint serves to let out any possible air bubbles which might have entered the tube while filling it with mercury The side arm is connected through rubber tubing to the leveling bulb A stopcock with a 3 mm bore connects the vertical arm to the manometer and the chamber through a screw-clamped rubber tubing. The entire apparatus with the open manometer is mounted on a board This board is attached in vertical position to the edge of a table near a water tap A hole is cut in the board to permit easy rotation of the extraction chamber

A water motor or any motor with 200-300 rpm can be used for shaking The chamber is shaken by means of a metal rod connected to an eccentric wheel of the motor on one side and a clamp on the water jacket on the other

For cleaning the mercury is removed and the apparatus is filled with cleaning solution then washed thoroughly with water and dried with acetone Finally air is sucked through to remove all the acetone. Any rubber connections which have been in contact with cleaning

solution are removed and replaced. For an occasional cleaning of the extraction chamber (after several determinations a thin layer of precipitate may have formed on the walls and can not be removed by water or alkali) the chamber alone may be filled for a period of 12 or 24 hours with cleaning solution (dichromate-sulfuric acid). Care must be taken that no acid reaches the rubber connections below the gas chamber or the tubings. This can be achieved by regulating the position of the leveling bulb and by closing the stopcock b. The stopcock grease as described on page 28 is used but the stopcocks should not be removed and greased too often. The stopcocks should be cleaned by wiping them with clean lintfree towels. All openings of the apparatus should be plugged with cotton in order to keep the mercury clean.

*Test for vacuum.* The extraction chamber is filled up to the upper stopcock with mercury by lifting the leveling bulb to expel the air from the apparatus then the upper stopcock is closed and sealed with mercury and the bulb lowered until the mercury has reached the 50 ml mark. If a leak is present, air will be sucked in by frequent lifting and lowering of the bulb. In such a case air will prevent the mercury from reaching the upper stopcock when the bulb is raised. If the apparatus is airtight, the mercury will audibly hit the walls of the apparatus upon lifting of the bulb. An alternate method for testing for leakage is as follows: the extraction chamber is filled with an amount of water corresponding to the volume of the test solution. After 3 minutes shaking to remove the air the pressure is read on the manometer at a volume of 0.5 ml. The procedure is repeated, and if the apparatus is airtight the readings must be identical.

### *Reagents*

(1) N/10 lactic acid, prepared by diluting immediately before use a 5 N lactic acid solution (1:50). The dilute solution is easily contaminated by moulds. To the dilute solution 2-3 drops of caprylic alcohol are added and the mixture is well shaken before use.

*Procedure.* Drawing of the blood, separation of the plasma and its saturation with alveolar air are performed as described for the volumetric analysis. The apparatus, including the capillary pipet above the stopcock is filled with mercury. The stopcock is closed and the leveling bulb lowered (position 1). The negative pressure should be

very slight and the mercury in this position must not fall when the stopcock is closed. After a few milliliters of the lactic acid-caprylic alcohol mixture have been brought into the filling cup, 1 ml. of plasma is introduced into the extraction chamber, using the Ostwald pipet. The rubber tip of the pipet is pressed against the bottom of the cup and the plasma is sucked into the chamber by opening the upper stopcock of the apparatus and the stopcock of the pipet. When the plasma has reached the lower mark of the pipet the flow of the sample is interrupted by closing the stopcock of the pipet. Then the upper stopcock of the apparatus is closed and the pipet is removed simul-

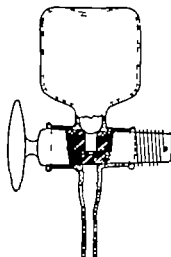


FIG. 11 APPARATUS FOR PRODUCING A MERCURY-SEALED STOPCOCK

taneously. Lactic acid from the filling cup is now allowed to flow into the chamber, until the liquid has reached the 2.5 ml. mark. The stopcock is closed and a drop of mercury is placed into the cup to secure a tight seal (fig. 11). The excess of lactic acid in the cup is removed by suction with a water pump.

It is recommended to insert a separatory funnel with a 2-hole rubber stopper between water pump and apparatus to collect any mercury which might be contained in the stream of lactic acid and which destroys the pipes by forming an amalgam. The mercury collects in the funnel from which it can easily be removed through the stopcock, washed and used again (fig. 12). After the lactic acid and the plasma have been mixed well by frequent raising and lowering of the bulb, the bulb is lowered until the surface of the mercury (not

of the liquid) has reached the 50 ml mark at the bottom of the chamber. The stopcock b is closed and the chamber is shaken for three minutes. Stopcock b is opened, the bulb raised and the volume of the gas is brought to the mark 2 (measured at the surface of the liquid, not of the mercury). This adjustment must be done carefully. It is advised to close the stopcock as soon as the liquid approaches the mark 2 and to regulate the final adjustment with the pinch clamp

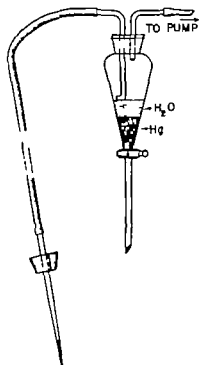


FIG 12 TRAP FOR MERCURY

(below stopcock b) Any abrupt flow of mercury to the chamber during the volume regulation after extraction has to be avoided because of possible reabsorption of the developed  $\text{CO}_2$ . If the adjustment has not been achieved immediately the mixture must be shaken for another minute and the volume regulation repeated as described above. The pressure  $P_1$  is read from the manometer, the stopcock b remaining closed. Now the mercury is removed from the filling cup by suction and the extracted gas is ejected from the apparatus by opening the stopcock beneath the cup and careful lifting of the bulb to a level above the chamber. Loss of liquid is to be avoided. A drop

of mercury is again placed on the bottom of the filling cup the surface of the liquid is brought to mark 2 and the pressure  $P_2$  is read from the manometer. The difference between  $P_1$  and  $P_2$  corresponds to the

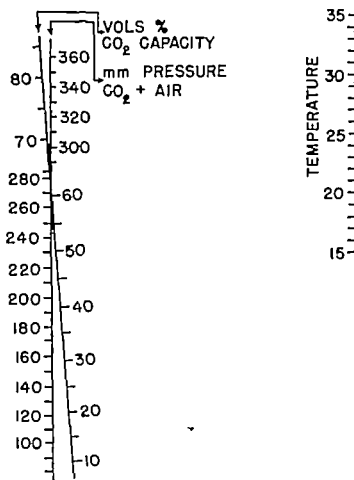


FIG 13 Nomogram (line chart) according to van Slyke and Neill. All gradations at right angles to their axes.

pressure of gas ( $\text{CO}_2$  plus air) ejected from 2.5 ml of liquid (plasma and lactic acid).

*Calculation.* With the aid of the van Slyke and Neill nomogram<sup>4</sup> (fig 13) the  $\text{CO}_2$  capacity can be calculated from these pressure readings and from the temperature. The value for the pressure difference  $P_1 - P_2$  is inserted into the left vertical line and the value for the temperature into the right vertical line of the nomogram. A line is drawn through these 2 points. The  $\text{CO}_2$  content (alkali reserve) can

be read exactly (to 0.5 per cent) at the intersection of this line with the slanting line of the nomogram

### *Example*

$P_1 = 42.5$  mm temperature  $24^\circ\text{C}$

$P_2 = 16.5$  mm

$D = 260$  mm.

From these figures an alkali reserve of 58 volume per cent can be read from the nomogram i.e. 100 ml serum at 40 mm  $\text{CO}_2$  pressure combine with 58 ml of  $\text{CO}_2$

## MANOMETRIC MICRO-DETERMINATION OF THE ALKALI RESERVE

### *Reagents*

N/100 lactic acid, prepared by diluting one ml of 5 N lactic acid to 500 ml. with water

### *Procedure*

Enough plasma is introduced into a 50 ml. separatory funnel for duplicate analysis. The plasma is saturated with air of 5.5 per cent  $\text{CO}_2$  content as described above. The apparatus is prepared as described on p. 35 and 0.2 ml. of plasma is brought into the chamber followed by lactic acid and caprylic alcohol up to mark 2. After the stopcock has been sealed off with mercury and the excess lactic acid removed from the filling cup, the liquids are well mixed by raising and lowering the leveling bulb, and the chamber is shaken for three minutes. The pressure  $p_1$  of the extracted gases is read at a volume of 0.5 ml. Then the gases are ejected from the chamber, the level is again adjusted to the 0.5 mark and the pressure  $p_2$  is read. The difference between  $p_1$  and  $p_2$  is recorded and the corresponding  $\text{CO}_2$  value is found from the chart as described for the macro-determination. Since the table has been computed for the semi micromethod, the result has to be multiplied by 1.25.

This factor is arrived at by the following calculation. If the sample size used for the micromethod is transferred to the semi micromethod, the results must be multiplied by 5 (1 ml./0.2 ml.). Since in the micromethod the volume has been reduced one-fourth (0.5 ml./2 ml.) the resulting figure must be divided by 4. This would result in a

of mercury is again placed on the bottom of the filling cup the level of the liquid is brought to mark 2 and the pressure  $P_2$  is read on the manometer. The difference between  $P_1$  and  $P_2$  corresponds

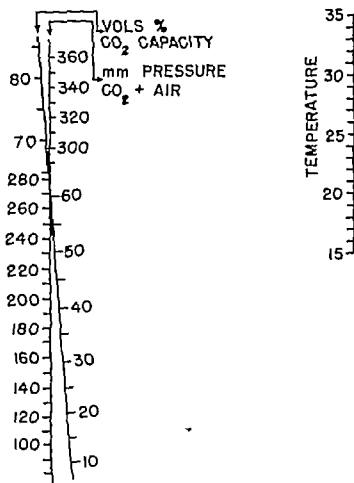


FIG. 13. Nomogram (line chart) according to van Slyke and Neill. The gradations are at right angles to their axes.

pressure of gas ( $\text{CO}_2$  plus air) ejected from 2.5 ml. of liquid (blood and lactic acid).

**Calculation.** With the aid of the van Slyke and Neill nomogram (fig. 13) the  $\text{CO}_2$  capacity can be calculated from these pressures and from the temperature. The value for the pressure difference  $P_1 - P_2$  is inserted into the left vertical line and the value for the temperature into the right vertical line of the nomogram.

be read exactly (to 0.5 per cent) at the intersection of this line with the slanting line of the nomogram

### *Example*

$P_1 = 425$  mm temperature  $24^\circ\text{C}$

$P_2 = 165$  mm

$D = 200$  mm

From these figures an alkali reserve of 58 volume per cent can be read from the nomogram i.e. 100 ml. serum at 40 mm  $\text{CO}_2$  pressure combine with 58 ml. of  $\text{CO}_2$ .

## MANOMETRIC MICRO-DETERMINATION OF THE ALKALI RESERVE

### *Reagents*

N/100 lactic acid, prepared by diluting one ml. of 5 N lactic acid to 500 ml. with water

### *Procedure*

Enough plasma is introduced into a 50 ml. separatory funnel for duplicate analyses. The plasma is saturated with air of 5.5 per cent  $\text{CO}_2$  content as described above. The apparatus is prepared as described on p. 35 and 0.2 ml. of plasma is brought into the chamber followed by lactic acid and caprylic alcohol up to mark 2. After the stopcock has been sealed off with mercury and the excess lactic acid removed from the filling cup the liquids are well mixed by raising and lowering the leveling bulb and the chamber is shaken for three minutes. The pressure  $p_1$  of the extracted gases is read at a volume of 0.5 ml. Then the gases are ejected from the chamber the level is again adjusted to the 0.5 mark and the pressure  $p_2$  is read. The difference between  $p_1$  and  $p_2$  is recorded and the corresponding  $\text{CO}_2$  value is found from the chart as described for the macro-determination. Since the table has been computed for the semi-micromethod, the result has to be multiplied by 1.20.

This factor is arrived at by the following calculation. If the sample size used for the micromethod is transferred to the semi-micromethod the results must be multiplied by 5 (1 ml. / 0.2 ml.). Since in the micromethod the volume has been reduced to one-fourth (0.5 ml. / 2 ml.)



factor of  $5/4 = 1.25$ , if the corrections for unextracted and redissolved  $\text{CO}_2$  were the same for semi micro and microanalysis. However, the extraction is more complete with a small volume of solution whereas the reabsorption amounts to 37 per cent, as compared to 17 per cent for the semi micromethod. Under these circumstances the empirically developed factor 1.20 for the multiplication of the microvalues gives results which are in good agreement with the results obtained with the semi micromethod.

Use of pipets (see pp. 31, 33)

### Example

$p_1 = 375$  mm temperature  $20^\circ\text{C}$

$p_2 = 163$  mm

$D = 212$  mm.

According to the nomogram these figures will represent an alkali reserve of 45.5 which multiplied by the factor 1.20 results in the final value of 57.33 vol. per cent  $\text{CO}_2$ .

Normal values for the alkali reserve are 55–80 vol. per cent  $\text{CO}_2$  for adults and 43–65 vol. per cent  $\text{CO}_2$  for children.

The alkali reserve varies considerably under physiologic conditions. After food intake the  $\text{CO}_2$  value rises and the alkali reserve drops, beginning approximately one hour after the meal and lasting for several hours. These changes are absent in hypoacidity of the gastric juice and are more marked in hyperacidity. During sleep the bicarbonate content of the blood rises, this rise is not connected with any change in the constituents of the blood, but is caused by a change in stimulation of the respiratory center. During heavy physical work the alkali reserve falls because the bicarbonate is used up as buffer for the lactic acid formed during exercise.

Respiration plays the biggest part in regulating the alkali reserve. Variations in the acid base balance can be compensated by changes in the depth of respiration. During reduced ventilation of the lung an increase in alveolar  $\text{CO}_2$  tension occurs followed by an increase of bicarbonate according to the formula



This condition (increased alkali reserve) is encountered in primary asphyxia, in emphysema, cardiac lung, pneumonia also as a result of

an alkalinizing diet (vegetables, potatoes), and medication (bicarbonate, magnesia, etc.)

In cases of increased ventilation of the lung a decrease in alkali reserve is found e.g. in fever, encephalitis high external temperature, etc.

A decreased alkali reserve can also be found in all processes accompanying increased acid formation in the organism, i.e.

- (1) diabetes mellitus, precomatose 30-40 vol. per cent  $\text{CO}_2$  during coma values to and below 15 vol. per cent  $\text{CO}_2$
- (2) in kidney diseases terminal state of nephritis, hydronephrosis, pyonephrosis cystic kidney
- (3) eclampsia, toxic pregnancy, hyperemesis gravidarum.
- (4) during starvation (formation of ketobodies)
- (5) hyperacidity of the stomach
- (6) ingestion of acids, acid-poisoning
- (7) excess ingestion of ammonia salts (ammonium chloride, phosphate etc)
- (8) acid diet (meat, cereals, etc.)

#### MANOMETRIC SEMIMICRO-METHOD FOR THE DETERMINATION OF BLOOD GASES (CARBON DIOXIDE, OXYGEN, CARBON MONOXIDE NITROGEN)

*Apparatus* see manometric determination of the alkali reserve, p. 35

#### *Principle of the method*

The blood gases are liberated in vacuum and the various gases are absorbed by specific reagents. The resulting differences in pressure represent the amounts of the various gases present.

#### *Reagents*

- (1) Potassium ferric cyanide-lactic acid mixture \*

(a) 3.3 Gm. of potassium ferric cyanide 3.3 Gm. of saponin 450 Gm. of urea 5 ml. of caprylic alcohol are dissolved in distilled water in a 1 liter volumetric flask and made up to the mark.

(b) N lactic acid made up fresh each time by diluting a 5 N lactic acid solution (see determination of alkali reserve). Before use 6 ml. of solution a are mixed with 0.66 ml. of solution b in the filling cup of the apparatus.

(2) 5 N NaOH (20 per cent), or N/1 NaOH

(3) Sodium hyposulfite reagent 10 Gm of sodium hyposulfite and 1 Gm of sodium anthraquinone- $\beta$ -sulfonate are dissolved in 50 ml of 0.5 N sodium hydroxide in a beaker and filtered rapidly through cotton (if sodium anthraquinone- $\beta$ -sulfonate is not available and the determination has to be carried out without the use of it, the oxygen absorption is retarded. It catalyzes the absorption of  $O_2$ . The absorption is completed in one minute with the mixture, otherwise 3-4 minutes would be required by hyposulfite alone). The filtrate is directly brought into the apparatus in order to remove any traces of dissolved air. The upper stopcock is closed and sealed with mercury. The chamber is now evacuated by lowering the mercury level in the pipet to the 50 ml mark and the chamber is shaken for 2-3 minutes. The liberated gas is ejected from the chamber by raising the mercury and opening the upper stopcock, the stopcock is closed and the evacuation is repeated. This gas free solution may be kept for a short period of time (one day) in a calcium chloride tube (fig 14a), which is equipped with a short rubber tubing with a pinch clamp and a small glass tube. Paraffin is used to protect the liquid from contact with air. No air must come in contact with the reagent during its transfer from the chamber to the calcium chloride tube. Several drops of paraffin are placed in the tube. The pinch clamp is opened to allow paraffin to fill the rubber tubing and the glass tube. By raising the leveling bulb the manometer reservoir is filled partly with mercury to establish a high pressure in the apparatus. Then the stopcock b is closed and a rubber tipped glass tube (as described for the pipet) is pressed against the bottom of the filling cup. By careful opening of the stopcock a and the pinch clamp of the calcium chloride tube the reagent is forced into the tube by the mercury pressure. When all of the hyposulfite has been removed from the chamber, the stopcock and pinch clamp are closed simultaneously. This reagent must be prepared fresh every day. The sodium hydroxide (5 N and N/1) used for the  $CO_2$  absorption are freed from air in a similar manner.

Large amounts of absorption reagent may be kept free from gas in a modified Haempel pipet (fig 14b).

(3) pyrogallol if only occasional oxygen analyses are required a pyrogallol solution may be used instead of the bisulfite. Pyrogallol

will keep indefinitely. 300 Gm. potassium hydroxide (alcohol free, purest grade) are dissolved in 200 ml. of water and introduced into a glass stoppered bottle beneath a deep layer of paraffin. For each 100 ml. of alkali 15 Gm. of pyrogallol are added. This blue-green solution turns deep brown when oxygen is added.

**Preparation of the blood sample.** The blood is taken with a dry or parafinated syringe and transferred to a test tube containing a few

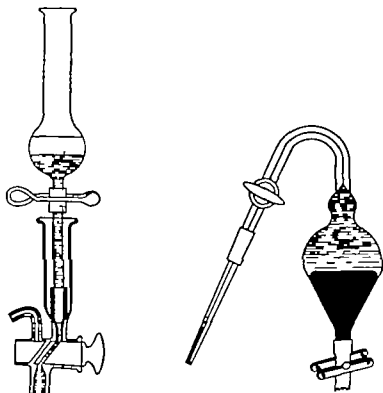


FIG. 14 Apparatus for keeping reagents free from gas.

ml. of mineral oil and a small amount of a mixture consisting of 2 parts lithium or potassium oxalate and 1 part sodium fluoride. The fluoride prevents enzymatic degradation of the carbohydrates (lactic acid formation).

**Procedure.** After the apparatus has been cleaned and tested for vacuum 6 ml. of the ferricyanide-lactic acid reagent are brought into the reaction chamber. The upper stopcock is closed, sealed off with mercury and the reagent is freed from air by shaking in vacuo (mercury to the 50 mark). The evacuation must be repeated until con-

stant readings are obtained on the manometer. Then the reagent is transferred to the filling cup by careful raising of the leveling bulb. One ml. of blood which has been thoroughly mixed under mineral oil, is transferred with an Ostwald pipet into the extraction chamber as described before and the reagent is added to the 3.5 mark. The upper stopcock is closed, sealed with mercury, the excess reagent is removed by suction from the cup and the cup is washed with water. The contents of the chamber are well mixed by repeated rising and lowering of the bulb. Finally the bulb is lowered until the mercury meniscus in the chamber has reached the 50 mark, the stopcock b is closed and the chamber is shaken for three minutes. By opening the stopcock b and lifting the leveling bulb the meniscus of the liquid is brought to the 2 mark. The liquid level should rise slowly. Any jerking motions of the mercury in the chamber during the volume reduction after extraction should be avoided since reabsorption of the gases may take place under these conditions, should this occur, the process of evacuation shaking and adjustment to the mark must be repeated. The total pressure  $p_1$  of all the ejected gases is read from the manometer.

For the determination of  $\text{CO}_2$ , 1 ml. of gas-free N/1 NaOH or 0.2 ml. of 5 N NaOH plus the volume of the stopcock bore are measured into the filling cup (the volume of the stopcock bore is usually marked on the apparatus by an arrow on the filling cup). The alkali is let into the chamber in several portions using slight vacuum. The upper meniscus of the liquid should approximately reach the mark 4. Care must be taken not to allow any air to enter the chamber. The stopcock is again sealed off with mercury and the liquid in the chamber is mixed by careful shaking by hand. The  $\text{CO}_2$  is thus absorbed, the liquid meniscus is adjusted to the 2 mark and the pressure  $p_2$  (gas pressure after absorption of  $\text{CO}_2$ ) is read from the manometer. For the determination of oxygen 1 ml. of the air-free hyposulfite solution (3) is brought into the chamber in small portions using low vacuum (see  $\text{CO}_2$  absorption). The chamber is gently shaken by hand, the liquid level adjusted to the 2 mark and the pressure  $p_3$  is read from the manometer. The sensitivity of the manometer can be altered by changing the volume of the liberated gases. By decreasing the gas volume the sensitivity of the determination can be increased according to the law of Boyle-Mariotte—the gas pressure is inversely propor-

tional to the volume. If the gas is adjusted to a volume of 2 ml 0.01 ml of this gas corresponds to a manometer reading of 4 mm. When this same gas volume is brought to the 0.5 ml. mark, the manometer reading will be 16 mm. Should the difference  $p_1 - p_2$  be too small, the 0.5 mark may also be used for adjustment and the pressure corresponding to this point should be read.

After the absorption of the oxygen a small constant gas residue remains (1.2-1.4 vol per cent). This residue represents the nitrogen. If the blood sample contains carbon monoxide, the pressure  $p_2$  is read and the gas left over after  $\text{CO}_2$  and  $\text{O}_2$  have been absorbed is ejected from the chamber and the liquid is adjusted to the 2 or 0.5 mark. The difference  $p_2 - p_4$  shows the amount of  $\text{CO} + \text{nitrogen}$ . The  $\text{CO}$  content is obtained by subtraction of 1.2 vol. per cent ( $\text{N}_2$ ). If only  $\text{CO}_2$  and  $\text{O}_2$  are to be determined the procedure can be simplified as follows. The  $\text{CO}_2$  is determined by absorption ( $p_1 - p_2$ ) and the  $\text{O}_2$  and  $\text{N}_2$  content are obtained together by ejecting the gases from the chamber. By subtraction of 1.2 volume per cent ( $\text{N}_2$  content) the oxygen content of the sample is calculated.

If only the carbon monoxide is to be determined, no separate absorption of  $\text{CO}_2$  by sodium hydroxide is needed. The  $\text{CO}_2$  can then be absorbed by bisulfite together with the oxygen. The gas which remains behind after absorption with sodium hyposulfite (or pyrogallol) is a mixture of  $\text{CO}$  and nitrogen. By ejection of these gases from the chamber the pressure  $p_3$  is determined and by subtraction of the nitrogen factor of 1.2 vol per cent the  $\text{CO}$  content of the blood is found.

### Calculation

#### (1) calculation of $\text{CO}_2$

$$P_{\text{CO}_2} = p_1 - p_2 - P_{\text{CO}_2}$$

$p_1$  = pressure of the gases after treatment with potassium ferricyanide lactic acid and urea mixture.

$p_2$  = pressure after absorption of  $\text{CO}_2$  with  $\text{NaOH}$

$P_{\text{CO}_2}$  = a constant (see below)

The difference expressed in mm Hg, multiplied by the factor indicated in table 4 gives the  $\text{CO}_2$  content in vol. per cent.

#### (2) Calculation of the oxygen

$$P_{O_2} = p_2 - p_3 - c_{O_2} \quad (a = 2 \text{ ml}) \text{ or}$$

$$P_{O_2} = p'_2 - p'_3 - c_{O_2} \quad (a = 0.5 \text{ ml.})$$

$p_2$  = pressure of the gases after absorption of  $CO_2$

TABLE 4—Factors for the calculation of gas content of blood ( $CO_2$ ,  $O_2$ ,  $CO$ ,  $N$ )

Temperature (°C)	Factor for $CO_2$			Factor for $O_2$ , $CO$ and $N$					
	Sample = 0.2 ml S = 2.0 ml a = 0.5 ml i = 1.03 ml	Sample = 1.0 ml S = 3.3 ml a = 2.0 ml i = 1.014 ml	Sample = 2.0 ml S = 7.8 ml a = 2.0 ml i = 1.00 ml	Sample = 0.2 ml S = 2.0 ml a = 0.5 ml i = 1.00 ml	Sample = 1 ml S = 3.3 ml		Sample = 2 ml S = 7 ml		
15	0.335	0.2725	0.1483	0.312	0.0523	0.2493	0.0317	0.1251	
16	0.333	0.2711	0.1470	0.310	0.0521	0.2485	0.0315	0.1246	
17	0.331	0.2697	0.1456	0.309	0.0519	0.2478	0.0314	0.1243	
18	0.330	0.2683	0.1449	0.303	0.0517	0.2468	0.0312	0.1237	
19	0.328	0.2669	0.1430	0.307	0.0515	0.2459	0.0311	0.1232	
20	0.327	0.2655	0.1429	0.307	0.0513	0.2450	0.0309	0.1228	
21	0.326	0.2640	0.1419	0.306	0.0510	0.2441	0.0308	0.1224	
22	0.324	0.2626	0.1410	0.305	0.0508	0.2432	0.0306	0.1219	
23	0.323	0.2613	0.1401	0.303	0.0506	0.2423	0.0305	0.1215	
24	0.322	0.2600	0.1391	0.302	0.0504	0.2414	0.0303	0.1210	
25	0.320	0.2588	0.1382	0.301	0.0502	0.2406	0.0302	0.1206	
26	0.318	0.2575	0.1373	0.300	0.0500	0.2398	0.0301	0.1202	
27	0.317	0.2562	0.1364	0.299	0.0598	0.2390	0.0299	0.1198	
28	0.316	0.2549	0.1356	0.298	0.0506	0.2382	0.0298	0.1193	
29	0.314	0.2537	0.1349	0.297	0.0503	0.2374	0.0296	0.1189	
30	0.313	0.2525	0.1341	0.296	0.0502	0.2366	0.0295	0.1185	
31	0.312	0.2515	0.1333	0.295	0.0500	0.2358	0.0294	0.1181	
32	0.311	0.2504	0.1325	0.294	0.0588	0.2350	0.0292	0.1177	
33	0.310	0.2493	0.1318	0.293	0.0586	0.2342	0.0291	0.1173	
34	0.308	0.2482	0.1310	0.292	0.0583	0.2333	0.0290	0.1169	

S = total volume of liquid.

a = mark chosen for gas

i = constant of apparatus.

$p_2$  = pressure after absorption of oxygen

$c_{O_2}$  = constant.

The difference in mm Hg multiplied by the factor indicated in the table results in the oxygen content in volume per cent.

If the reading was taken with the gas at the 0.5 mark instead of at the 2 ml. mark, the corresponding volume of the table must be used

### (3) Calculation of CO and N<sub>2</sub>

$$P_{H_2-CO} = p_3 - p_4$$

$p_3$  = pressure after absorption of oxygen and CO<sub>2</sub>

$p_4$  = manometer reading after ejection of the gas residue. We found that it is not necessary to apply a correction factor as no absorption liquid is introduced into the chamber

The CO content is obtained by subtracting 1.2 volume per cent from the above calculated value. The 1.2 volume per cent represents the nitrogen

The calculation with the table can only be made if the constants of the apparatus (volume of chamber 50 ml., volume of reagent mixture S = 3.5 ml. reading at the 0.5 ml. or 2 ml. mark 1 ml. blood sample) are identical with those of the table. Table derived from H. Schwarz, *Mikrogasanalyse*, Wien 1935

Introduction of a measured amount of absorbing agent (1 ml. of N/1 or 0.2 ml. of 5 N sodium hydroxide, 1 ml. of sodium hyposulfite, pyrogallol etc.) into different apparatus results in different pressure drops in the manometer. Therefore a corresponding factor has to be determined for each apparatus. This constant is determined by a blank analysis as follows: 5 ml. of potassium ferricyanide-lactic acid reagent are evacuated till the liquid is gas-free (constant manometer readings after ejection of the liberated gases). For the following analysis only 3.5 ml. of reagent is used then an equal amount of sodium hydroxide (1 ml. of N/1 or 0.2 ml. of 5 N), hyposulfite (1 ml.), pyrogallol (1 ml.) is added and the pressure fall after each addition is noted. This constant varies between 0.5 and 4.0 mm Hg.

Example of a semi-microdetermination of CO<sub>2</sub>, O<sub>2</sub>, and N<sub>2</sub> with absorption of CO<sub>2</sub> and O<sub>2</sub>

		Temperature 22° C			
P	mark 2	mark 0.5			
$p_1$	420.1		CO <sub>2</sub>		1.4
$p_2$	228.7	381.8	CO <sub>2</sub>	(a = 2 ml.)	1.6
$p_3$	178	156	CO <sub>2</sub>	(a = 0.5 ml.)	2.4
$p_4$	173	165			

$$\text{vol per cent CO}_2 = (p_1 - p_2 - c_{\text{CO}_2}) \times F = (420.1 - 228.7 - 1.4) \times F = (191.4 - 1.4) \times F = 190 \times 0.2626 = 49.89$$



$$\text{vol. per cent O}_2 (a = 2 \text{ ml}) = (p_2 - p_1 - c_{\text{O}_2}) \times F = 223.7 - 178 - 1.6 \times F = (50.7 - 1.6) \times F = 49.1 \times 0.2432 = 11.94$$

$$\text{vol. per cent O}_2 (a = 0.5 \text{ ml parallel microanalysis}) = (p_2 - p_1 - c_{\text{O}_2}) \times F = (384.8 - 186 - 2.4) \times F = 196.8 - 2.4 \times F = 196.4 \times 0.0608 = 11.94$$

$$\text{vol. per cent N}_2 (a = 2 \text{ ml}) = (p_2 - p_4) \times F = (178 - 173) \times F = 5F = 5 \times 0.2432 = 1.21$$

$$\text{vol. per cent N}_2 (a = 0.5 \text{ ml}) = (p_2 - p_4) \times F = (186 - 105) \times F = 81 \times 0.0608 = 1.276$$

Example of a CO<sub>2</sub> and O<sub>2</sub> analysis without absorption of the O<sub>2</sub>

P	Temperature 24° C		Cco <sub>2</sub> = 1.4
	mark 2	mark 0.5	
p <sub>1</sub>	430		
p <sub>2</sub>	215.5	338	
p <sub>3</sub>	173	165	

$$\text{vol. per cent CO}_2 = (p_1 - p_2 - c_{\text{CO}_2}) \times F = (430 - 215.5 - 1.4) \times F = (214.5 - 1.4) \times F = 213.1 \times F = 213.1 \times 0.2600 = 55.40$$

$$\text{vol. per cent O}_2 + \text{N}_2 = (215.5 - 173) \times F = 42.5 \times F = 42.5 \times 0.2414 = 10.26 (a = 2 \text{ ml})$$

$$\text{vol. per cent O}_2 = 10.26 - 1.2 = 9.06$$

$$\text{vol. per cent O}_2 + \text{N}_2 = (338 - 165) \times F = 173 \times 0.0604 = 10.45 (a = 0.5 \text{ ml})$$

$$\text{vol. per cent O}_2 = 10.45 - 1.2 = 9.25$$

Example of a CO analysis with previous absorption of CO<sub>2</sub> and O<sub>2</sub> together

P	Temperature 20° C	
	mark 2	mark 0.5
p <sub>1</sub>	438.5	
p <sub>2</sub>	198.0	268
p <sub>3</sub>	173.0	166

$$\text{vol. per cent CO} + \text{N}_2 = (p_2 - p_3) \times F = (198 - 173) \times F = 25 \times 0.2450 = 6.125 (a = 2 \text{ ml})$$

$$\text{vol. per cent CO} = 6.125 - 1.2 = 4.925$$

$$\text{vol. per cent CO} + \text{N}_2 = (p_2 - p_3) \times F = (268 - 166) \times F = 102 \times 0.0613 = 6.252 (a = 0.5 \text{ ml})$$

$$\text{vol. per cent CO} = 6.252 - 1.2 = 5.052$$

If only the oxygen bound by the hemoglobin is to be determined the value corresponding to the dissolved oxygen has to be subtracted from the result. In venous blood 0.1 vol. per cent  $O_2$  must be deducted in arterial blood 0.2 vol. per cent  $O_2$ .

#### MANOMETRIC MICRO-METHOD FOR THE DETERMINATION OF THE BLOOD GASES ( $O_2$ , $CO_2$ , $N_2$ )

Reagents and preparation of the blood sample as described above for the semi-micromethod

##### *Procedure*

An Ostwald pipet with a 0.2 ml. mark below the stopcock is used to deliver 0.2 ml. of blood. The pipet is filled first with approximately 1 ml. of Hg by a mercury pump then with 0.2 ml. of blood to the mark. The stopcock of the pipet is closed. If no mercury pump is available the blood gas apparatus can be used as follows. The pipet is connected with rubber tubing to the glass tube above stopcock (a) and with stopcock (b) closed the leveling bulb is lowered, drawing mercury from a reservoir into the pipet.

After the potassium ferric-cyanide-lactic acid reagent has been freed from air in the apparatus and has again been brought into the filling funnel the blood is allowed to flow slowly into the chamber, and the pipet is washed with mercury. The ferricyanide reagent is let in to the 2 mark. After mixing the blood gases are liberated by shaking for three minutes and the pressure is read at a volume of 0.5 ml. ( $p_1$ ). Adjustment of the liquid is done as described for the macro-analysis.  $CO_2$  is absorbed by 0.1 ml. of 5 N NaOH (plus additional NaOH to account for the volume of the stopcock bore) or 0.5 ml. of N/1 NaOH (gas-free) and the pressure ( $p_2$ ) is determined. For the absorption of oxygen 0.5 ml. of hyposulfite solution is used and the pressure ( $p_3$ ) is read from the manometer. As described in the semi-micromethod the oxygen determination by absorption with hyposulfite or pyrogallol may be omitted and the  $O_2$  can be calculated by subtraction of the  $N_2$  constant (1.2 vol. per cent) from the gas residue.

*Calculation* From the three figures corresponding to  $p_1$ ,  $p_2$ ,  $p_3$  the values for  $CO_2$ ,  $O_2$ , CO and  $N_2$  are calculated as described for the semi-micromethod. Blank runs must be carried out with the suitable reagents to obtain the constants  $c$ .

Example of a microanalysis  $\text{CO}_2$ ,  $\text{O}_2$ ,  $\text{CO}$  and  $\text{N}_2$

Temperature 21 °C		
P	a - 0.5 ml	
$p_1$	380.1	$\text{Cco}_2 = 1.5 \text{ mm.}$
$p_2$	228.6	$\text{Co}_2 = 1.9 \text{ mm.}$
$p_3$	184.9	
$p_4$	164.2	

$$\text{vol per cent CO}_2 = (p_1 - p_2 - c_{\text{co}_2}) \times F = 380.1 - 228.6 - 1.5 \\ \times F = (151.5 - 1.5) \times F = 150 \times 0.3200 = 48.90$$

$$\text{vol per cent O}_2 = (p_2 - p_3 - c_{\text{o}_2}) \times F = (228.6 - 184.9 - 1.9) \times \\ F = (43.7 - 1.9) \times F = 41.8 \times F = 41.8 \times 0.306 = 12.79$$

$$\text{vol per cent CO} + \text{N}_2 = (p_3 - p_4) \times F = (184.9 - 164.2) \times F \\ = 20.7 \times 0.306 = 6.334$$

$$\text{vol per cent CO} = 6.334 - 1.2 = 5.134$$

#### SEMICRO-METHOD FOR THE DETERMINATION OF THE TOTAL OXYGEN CAPACITY<sup>6, 7</sup>

In the chamber of the van Slyke manometric apparatus a part of the blood sample prepared as described above is saturated with oxygen from the air and the oxygen thus absorbed is determined. A large correction has to be applied in this method for the physically dissolved oxygen. This correction factor can be calculated from the temperature and the barometric pressure.

#### Reagents

- (1) 0.9 per cent sodium chloride solution
- (2) Potassium ferricyanide-saponin reagent. 32 Gm. of potassium ferricyanide and 8 Gm. of saponin are dissolved in water and diluted to 100 ml. in a volumetric flask.
- (3) 5 N NaOH or N/1 NaOH (see above)
- (4) Air-free sodium hyposulfite solution (Preparation see p. 46)

#### Procedure

The chamber of the van Slyke apparatus is washed repeatedly with distilled water to remove any traces of acid or alkali (test with litmus paper), rinsed with 0.9 per cent NaCl solution and filled completely with mercury. Then 3 ml. of 0.9 per cent NaCl solution is placed in the cup of the apparatus, the upper stopcock is opened and about 0.5

ml. of NaCl solution is let into the chamber using low vacuum. As described above 1 ml. of blood is transferred to the chamber with an Ostwald pipet and the cup is washed with enough NaCl solution to reach the 3.5 mark. The excess NaCl solution is removed by suction with a water pump. The mercury is lowered in the chamber to the 50 ml. mark, its upper stopcock opened to the air and the chamber is shaken for approximately three minutes to saturate the hemoglobin quantitatively with oxygen. After the shaking a few drops of octyl alcohol (or caprylic alcohol) are added to the contents of the chamber to remove air bubbles from the glass walls and to settle the foam. By raising the leveling bulb the liquid level is brought to the height of the stopcock capillary to expel all air from the chamber. During this process no blood must enter the stopcock capillary, since the octyl alcohol, floating on the surface of the blood prevents the blood from leaving the chamber. Then 0.13 ml. of ferric cyanide solution (plus excess ferricyanide to account for the stopcock bore) is introduced into the apparatus. The volume of the bore is usually marked by an arrow on the filling funnel. After the stopcock has been closed and sealed with mercury the chamber is evacuated by lowering the mercury to the 50 ml. mark and the chamber is shaken under vacuum for three minutes. After the addition of 1 ml. of N/1 NaOH the  $\text{CO}_2$  is absorbed and after adjustment to the mark 2 the pressure  $p_1$  is read. Then 1 ml. of the air free alkaline hyposulfite reagent is added to the chamber as described above, the chamber carefully shaken by hand the level adjusted to the 2 mark and the pressure  $p_2$  is read.

### Calculation

$$\text{Volume per cent O}_2 = (p_1 - p_2 - C) \times F$$

$p_1$  = pressure of the liberated gases after absorption of  $\text{CO}_2$  by NaOH

$p_2$  = pressure after absorption of  $\text{O}_2$  by hyposulfite.

F = factor read from table 4

C = constant, including the following four components

$c_1$  = amount of oxygen dissolved in 2.5 ml. of 0.9 per cent NaCl

$c_2$  = amount of oxygen physically dissolved in 1 ml. of blood

$c_3$  = traces of oxygen contained in 0.13 ml. of saponin-ferric cyanide solution

$c_4$  = correction for the drop in the manometer after addition of 1 ml. of hyposulfite

The first two factors ( $O_2$  content of the NaCl solution and the oxygen dissolved in the blood) can be calculated from the nomogram (fig 15a) A straight line connecting the points for the barometric pressure and the temperature cuts the central scale at a point indicating the value of correction ( $c_1 + c_2$ ) Components 3 and 4 are determined in a

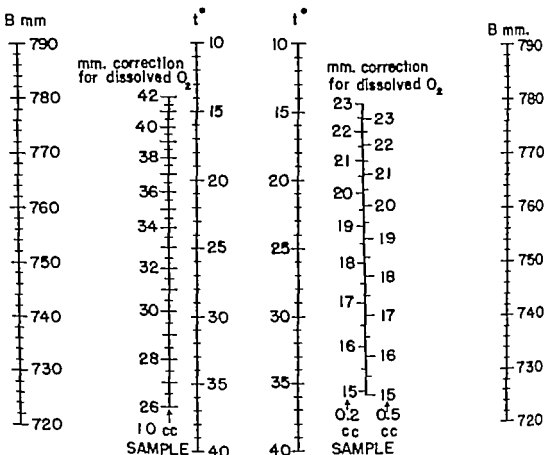


FIG 15 Nomograms for the calculation of total oxygen capacity (J Sendry Jr) a. For semimicromethod b. For micromethod

blank analysis as follows 3.5 ml of NaCl solution are rendered gas-free in the apparatus. The liberated gas is expelled and to this gas-free solution is added 0.13 ml of ferricyanide solution (as described above) It is again evacuated and the liberated O<sub>2</sub> absorbed with 1 ml of sodium hyposulfite The pressure drop in the manometer corresponds to the oxygen content of the potassium ferricyanide solution as well as to the correction for the sodium hyposulfite solution This drop of pressure is added each time to the factor read from

the nomogram (sum of  $c_3$  and  $c_4$ ) The value usually varies between 2.5 and 4 mm. This factor is constant for the same apparatus and the same ferricyanide solution

### Example

$a = 2$  ml.  $p_1 = 291$  mm (after absorption with NaOH)  
 Temperature  $22^\circ\text{C}$ .  $p_2 = 175$  mm (after absorption with hyp-  
 sulfite)

$c = c_1 + c_2 + c_3 + c_4$

$c_1$ and $c_2$ read from nomogram	35.2 mm.
$c_3$ and $c_4$	2.5 mm
	<hr/> 37.7 mm

Volume per cent oxygen  $= (p_1 - p_2 - C) \times F = (291 - 175 - 37.7) \times F = 78.3 \times 0.2432 = 19.04\%$

The total capacity i.e. the amount of oxygen bound chemically by 100 ml. of blood at maximal oxygen saturation is 19.04 ml.

### MICROMETHOD FOR THE DETERMINATION OF TOTAL OXYGEN CAPACITY

*Reagents* see under Semi-micromethod, p 54

### Procedure

One ml. of 0.9 per cent NaCl solution is placed in the filling cup and 0.2 ml. of blood is introduced into the chamber. The pipet is washed with NaCl solution which is added to the chamber. The cylinder is washed with small amounts of NaCl until the mercury meniscus has reached the 2 ml. mark. The excess of salt solution is removed by suction with the stopcock open to the air. The mercury is lowered to the 50 ml. mark and the blood-salt solution mixture is saturated with air by shaking for three minutes. Then the oxygen is determined as described above. The blood gases are liberated by the addition of 0.07 ml. of ferric cyanide-saponin solution (plus the volume of the stopcock bore) the  $\text{CO}_2$  is absorbed with 0.1 ml. of sodium hydroxide, and the oxygen with 0.5 ml. of sodium hyposulfite. The correction factor is determined as described for the analysis with 1 ml. but now the values must be taken from the nomogram (fig 15b). The calculation is done according to the formula

$(p_1 - p_2 - C) \times F = \% \text{ oxygen}$   $F$  is found in table 4

Example see under f)

### DETERMINATION OF HEMOGLOBIN AND CALIBRATION OF THE HEMOMETER ACCORDING TO SAHLI

The total oxygen capacity is directly proportional to the hemoglobin content. At 0°C and 760 mm Hg one gram of hemoglobin combines with 1.36 ml. of oxygen\*. In the absence of carbon monoxide the hemoglobin content of the blood sample can be calculated by dividing the total oxygen capacity by 1.36.

This method is very exact, but since the procedure is rather time consuming it can not be used at the bedside. Therefore, the hemoglobin content is determined colorimetrically by a rapid and simple method according to Sahli in a hemometer. 0.02 ml of blood is converted into acid hematin with N/10 HCl and this solution is diluted with water until the same color is obtained as in a standard hematin solution. The standard solution must be checked frequently unless Sahli's original hemometer is used.

#### Procedure

The hemoglobin is determined simultaneously according to Sahli and by determining the total oxygen capacity. If the sample is well standardized, one division of the hemometer scale corresponds to 0.16 gm of hemoglobin. Otherwise a correction factor must be used.

*Example* The sample shows a total oxygen capacity of 19.69 vol. per cent. From this value the hemoglobin content is calculated

$\frac{19.69}{1.36} = 14.47$  per cent. Hemometer value (Sahli) = 95. From this value would result a hemoglobin content of  $95 \times 0.160 = 15.20$  per cent. The figure read from the hemometer is too high and will give the correct value only by multiplication with the factor  $\frac{14.47}{15.20} =$

0.95. Until the next calibration all the values read on the hemometer have to be multiplied by 0.95.

### DETERMINATION OF HEMOGLOBIN AND OXYHEMOGLOBIN

The following example shows the calculation of the oxyhemoglobin and the reduced hemoglobin.

Total capacity	19.69 per cent
O <sub>2</sub> content of the blood taken anaerobically under mineral oil	<u>10.35 per cent</u>
	9.34 per cent

The difference corresponds to the non-reduced hemoglobin.

If 100 is the value for the total capacity (maximum saturation), the oxyhemoglobin content of the blood is calculated as follows

$$\frac{19.69}{10.34} = \frac{100}{x}$$

$$x = \frac{10.34 \times 100}{19.69} = 52.51 \text{ per cent}$$

the reduced hemoglobin content is calculated as follows

$$100 - y \text{ (} y = \text{oxygen content of oxyhemoglobin)} = 100 - 52.56 = 47.49 \text{ per cent}$$

In a normal person the total oxygen capacity of the blood averages 20 ml for each 100 ml of blood i.e. 20 vol per cent  $O_2$ . Higher values for the total oxygen capacity (up to 30 volume per cent or more) may be encountered during an increase in red cells (true polycythemia, or symptomatic polycythemia caused by decreased oxygen content of the atmospheric air). In anemic patients with a decrease in red cells and a lowered hemoglobin the blood is incapable of binding much oxygen and the total oxygen capacity of the blood may drop to 10 volumes per cent or less. This is also the case in states of blood dilution such as occur in nephritis with hydermia.

In a normal person the circulating hemoglobin is saturated with oxygen in the lungs, so that in the arterial blood about 95 per cent of the amount of hemoglobin corresponding to the total capacity is saturated with oxygen. The arterial oxygen saturation can be lowered by physical labor and drop below the normal range i.e. 92 per cent or less. In nervous persons with accelerated superficial respiration a lack of oxygen in the arterial blood has been observed, corresponding to a saturation of 90 — 80 per cent and less. In these persons no change of air occurs in the deeper respiratory passages the partial oxygen pressure in the alveolar air is decreased and the hemoglobin is incompletely saturated with oxygen.

In pathologic lung changes, such as emphysema, some cases of pneumonia, extensive damage from tuberculosis the oxygen saturation of the blood may drop considerably below the normal level. The same is found in some decompensated heart diseases; carbon monoxide poisoning and phosgene poisoning are some of the toxic states that lead to decreased arterial oxygen saturation.

In moderate cases of phosgene poisoning the lack of oxygen will not be severe during rest, but is considerable during physical work.



The arterial blood loses only part of its oxygen by passing through the tissues. The oxygen saturation of venous blood varies in different parts of the circulatory system and in the antecubital vein of a normal resting person it is between 60 and 70 volumes per cent. In patients with cyanotic heart diseases with circulatory disturbances the percentage of saturation in the venous blood drops to 50 volume per cent or less.

### DETERMINATION OF THE OXYGEN METABOLISM OF RED BLOOD CELLS<sup>10</sup>

In contrast to young red cells without nuclei, which possess very active gas metabolism, the normal older erythrocytes without nuclei have only a very small metabolic rate. This process can easily be demonstrated and measured quantitatively.

#### *Procedure*

Sterile venous blood is defibrinated by shaking with glass beads in a sterile flask for fifteen minutes and at the same time saturated with oxygen. In one part of the sample the oxygen content is determined according to one of the methods described above (semi micro or micro method). The remainder of the sample is transferred under mineral oil into a test tube, the tube is stoppered and left in an incubator at 37°C for five hours. During this time the red cells will settle down and the sample must be well mixed with a glass rod before analysis. Care must be taken to avoid all contact with air. The oxygen content of this sample is then determined. The difference between the oxygen content of the last and the first sample corresponds to the oxygen metabolism of the erythrocytes.

#### *Example*

O <sub>2</sub> content of the defibrinated blood (determined immediately)	5.8 ml
O <sub>2</sub> content of the defibrinated blood after incubation	1.8 ml

$$\text{difference} = 4 \text{ ml.} = 69 \text{ per cent } \left( \frac{5.8}{4} = \frac{100}{x} \right)$$

In this case the oxygen metabolism amounts to 69 per cent (in five hours)

The loss of oxygen in the blood of normal adult persons is up to

4 per cent, in anemia with active regenerative processes losses up to 70 per cent may be found. This method is advantageous because it provides a quantitative measurement of the regeneration and it indicates any regenerative process even in cases where it can not be demonstrated histologically. The results are only valid under strictly sterile conditions. The respiration of bacteria, tissue cultures and living cells can also be determined by this method.<sup>11</sup>

#### DETERMINATION OF THE pH IN SERUM AND PLASMA

*Principle of the method* If the  $\text{CO}_2$  tension of a mixture of sodium bicarbonate and  $\text{CO}_2$  (like that occurring in plasma or serum) alters, the amount of free  $\text{CO}_2$  changes at the cost of combined  $\text{CO}_2$ . It is either increased or decreased. This is connected with a fluctuation of pH.

According to Hasselbach<sup>12</sup> the pH can be calculated from the ratio of free to combined  $\text{CO}_2$ , as follows

$$\text{pH} = p_k + \log \delta + \log \frac{\text{BHCO}_3 (\text{in vol. } \%) }{\text{H}_2\text{CO}_3 (\text{in mm Hg})} \quad (\text{equation 1})$$

$k$  = dissociation constant of  $\text{H}_2\text{CO}_3$

$p$  = its negative logarithm

$\delta$  = degree of dissociation of bicarbonate. Since  $\delta$  varies between 0 and 0.1 it may be neglected entirely and according to Sendroy and van Slyke<sup>13</sup>  $p_k + \log \delta = p_{k1}$  the value 6.10 is used

$$\text{pH} = 6.10 + \log \frac{\text{BHCO}_3}{\text{H}_2\text{CO}_3} \quad (\text{equation 2})$$

$$\text{H}_2\text{CO}_3 (\text{free acid}) = \frac{p\alpha}{760} = \frac{0.510 \times p}{760} = 0.067 p$$

$p$  = partial pressure of  $\text{CO}_2$

$\alpha$  = absorption coefficient of  $\text{CO}_2$  at  $38^\circ\text{C}$ . (In serum this corresponds to  $\alpha = 0.510$ )<sup>14</sup>. Since the combined  $\text{CO}_2$  ( $\text{BHCO}_3$ ) = total  $\text{CO}_2$  in volume per cent - free  $\text{CO}_2$  in volume per cent the final equation results

$$\text{pH} = 6.10 + \log \frac{\text{vol. per cent } \text{CO}_2 - 0.037 \times p}{0.037 \times p} \quad (\text{equation 3})$$

*Determination of the pH in Venous Plasma (Serum)**Reagents*

(1) as an anticoagulant a mixture of 1 part of ammonium or sodium fluoride (exactly neutral) and 3 parts of potassium or lithium oxalate (neutral) is used

(2) Liquid paraffin

(3) N/10 lactic acid, gas-free. Six ml of N/10 lactic acid plus a few drops of octyl or caprylic alcohol are introduced into the gas chamber of the van Slyke apparatus and the absorbed gases are liberated by shaking in vacuo for three minutes. The gases are ejected from the chamber and the procedure is repeated until the manometer readings are constant (no more gas is liberated)

*Procedure*

Blood is taken from a nontourniqueted vein under strictly anaerobic conditions with a dry or paraffinated syringe and transferred under oil to a test tube containing a trace of the anticoagulant reagent. The plasma is rapidly separated from the cells and kept under oil for the determination which should be performed soon after the plasma has been separated. Three analyses are carried out on this sample

(1) In one part of the sample the total amount of  $\text{CO}_2$  is determined according to the semimicro- or micro-method (see pp 35-43). The lactic acid used must be freed from gas and kept in the cylinder of the apparatus. The  $\text{CO}_2$  is absorbed with N/1 NaOH or 5 N NaOH (gas-free) (see p 45)

(2) A second part of the sample is saturated with  $\text{CO}_2$  at 30 mm. Hg and  $38^\circ\text{C}$

(3) The third part of the sample is saturated with  $\text{CO}_2$  at 60 mm. Hg and  $38^\circ\text{C}$

For the saturation of the serum with  $\text{CO}_2$  of a desired tension the apparatus as shown in fig 16 is used \* 2.5 ml or 0.5 ml of plasma is introduced into a flask (saturator) of 100 times its volume. The flask is closed with a 2-hole rubber stopper. A capillary passes through one hole down to the bottom of the flask. At the free end this capillary is closed with rubber tubing and a pinch clamp. There is a short capillary in the second hole. It is connected to a vacuum

\* See also D. D. van Slyke and co-workers J Biol Chem 54 121 1922.

pump Between the capillary and the pump are connected a mercury manometer and a glass tube with sidearm which can be closed with a stopcock or a clamp The diameter of the manometer tube should be 4 mm. and the diameter of the mercury reservoir should be 10 times greater Since the cross section of the reservoir is 100 times larger than that of the manometer a rise of 1 mm. in the tube corresponds to a fall of 0.01 mm. in the reservoir In order to calculate the pressure change in the entire system, the pressure found must be

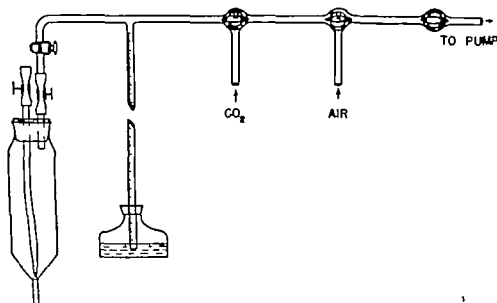


FIG 16 Apparatus for the saturation of serum (plasma) with CO<sub>2</sub> of desired tension

multiplied by 101 When the desired vacuum is reached the pump is switched off and it is waited for some time before proceeding with the analysis in order to note any variations in pressure due to leakage in the system. When the pressure remains constant a CO<sub>2</sub> developing Kipp-apparatus is connected with the sidearm the stopcock is opened CO<sub>2</sub> is introduced until the pressure has equilibrated and the clamp of the saturator is closed. Now the saturator is disconnected from the manometer and shaken for fifteen minutes to saturate the plasma with the CO<sub>2</sub>-air mixture. All this is performed at 38°C To remove the sample from the saturator (still at 38 C) the pipet is connected to the tubing which is attached to the long capillary After opening

of the pinch clamp the pipet fills up automatically as a result of a higher pressure in the flask caused by higher temperature (38°)

The  $\text{CO}_2$  tension of the plasma warmed to 38°C is calculated as follows

$$p_{2s} = 101 \times p_1 \times \frac{273 + 38}{273 + t} = 101 \times p_1 \times \frac{311}{273 + t} = p_1 \times \frac{314}{273 + t}$$

$p_{2s}$  =  $\text{CO}_2$  tension in the saturator

$p_1$  =  $\text{CO}_2$  tension at room temperature read from the manometer (without correction)

$t$  = room temperature

101 = above mentioned mercury correction factor for the fall in pressure on the manometer

The analysis in this sample is carried out in the same manner as in plasma. Gas-free lactic acid is used

The pH can be calculated from the  $\text{CO}_2$  tension and from the  $\text{CO}_2$  determined in the plasma according to equation 3

Example vol per cent  $\text{CO}_2$  in plasma calculated from

$p_1$	= 355.4	sample	= 1 ml
$p_2$	= 178	S	= 3.5 ml.
$C_{\text{CO}_2}$	= 1.4	a	= 2 ml
f	= 0.2655	t	= 20°C

$$1) \text{ vol per cent } \text{CO}_2 = (p_1 - p_2 - C_{\text{CO}_2}) \times f = 355.4 - 178 - 1.4 \times 0.2655 = 176 \times 0.2655 = 46.73$$

2) The sample has been saturated at 38°C with  $\text{CO}_2$  which has been introduced at a temperature of 20°C under a pressure of 28 mm Hg

$$p_{1s} = 28 \times \frac{314}{293} = 30 \text{ mm } \text{CO}_2$$

$V_{1\text{CO}_2}$  = 45 vol per cent  $\text{CO}_2$  (calculation  $\text{CO}_2 = (p_1 - p_2 - C_{\text{CO}_2}) \times f$ )

3) Sample shaken at 38° with  $\text{CO}_2$  which has been introduced at a temperature of 20°C under a pressure of 56 mm. Hg

$$p_{2s} = 56 \times \frac{314}{293} = 60 \text{ mm } \text{CO}_2$$

$V_{2\text{CO}_2}$  = 54 vol per cent (calculation see above)

The above obtained values for  $\text{CO}_2$  in vol per cent at 30 mm. Hg and 60 mm Hg tension are marked on the logarithmic chart of Peters<sup>15</sup> (fig. 17). The 2 points are connected with a straight line and the

CO<sub>2</sub> value in vol per cent for plasma is marked on this line. The pH is found by drawing a line parallel to the existing lines on the chart through the above determined CO<sub>2</sub> points. The corresponding CO<sub>2</sub>

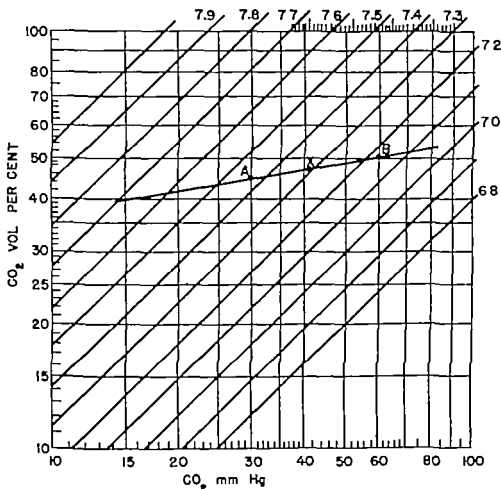


FIG. 17 Logarithmic chart for the calculation of pH in serum (plasma) (Peters)

tension in mm Hg can be read on the ordinate. In our sample the pH according to the figures is 7.33. If greater accuracy is desired the pH can be determined according to Hasselbach's formula from the CO<sub>2</sub> in plasma and from the pressure of 40 mm read from the logarithmic chart according to equation 3

$$\text{pH} = 6.10 + \log \frac{\text{vol. \% CO}_2 - 0.067 p}{0.037 p}$$

$$0.10 + \log \frac{40.73 - 0.07 \times 40}{0.07 \times 40}$$

$$0.10 + \log \frac{40.73 - 2.68}{2.68}$$

$$0.10 + \log \frac{44.05}{2.68}$$

$$0.10 + \log 16.43$$

$$0.10 + 1.2156 = 7.3156$$

### *Determination of the pH in Arterial Plasma*

When arterial blood (serum or plasma) is used for the analysis, the procedure is much simpler as only the total  $\text{CO}_2$  of the plasma, drawn under the above described precautions has to be determined, the  $p_{\text{CO}_2}$  is assumed to be constant (40 mm Hg). The  $\text{CO}_2$  content is determined by analysis and the pH can be read off directly from the van Slyke nomogram<sup>14</sup> (fig 18). The point for the  $\text{CO}_2$  content which is found on the left line of the nomogram is connected by a straight line with the constant  $\text{CO}_2$  tension (40 mm Hg) (right line) and at the point of intersection of the line with the center line the pH can be read. This value is called the reduced pH.

### *Example*

In the arterial plasma the determined volume per cent of combined and free  $\text{CO}_2$  are 50.5 volume per cent. From the nomogram reading the tension of 40 mm corresponds to a pH of 7.40.

The pH may also be calculated according to the Hasselbach formula (equation 3. See p. 73)

$$\text{pH} = 0.10 + \log \frac{\text{vol. \% CO}_2 - 0.007 p}{0.007 p}$$

$$0.10 + \log \frac{50.5 - 40 \times 0.007}{40 \times 0.007}$$

$$0.10 + \log \frac{50.5 - 2.68}{2.68}$$

$$0.10 + \log \frac{53.82}{2.68}$$

$$0.10 + \log 20.08$$

$$0.10 + 1.30 = 7.4$$

Only serum or plasma can be used in this method. In whole blood the hydrogen ion concentration and the  $\text{CO}_2$  combining power are dependent upon the oxygen saturation of the hemoglobin; the oxyhemoglobin acts as a weak acid. Therefore the pH of the whole blood cannot be determined by these methods.

The pH is normally kept constant by the entire organism. It is maintained by the process of ventilation (decreased or increased exhalation of  $\text{CO}_2$  by the lung) and by the neutralizing action of basic metabolism products ( $\text{NH}_3$ ) and excretion through the intestinal tract and the kidneys (partly also in perspiration).

The pH of the arterial plasma of a normal resting person is between 7.38 and 7.40. During voluntary hyperventilation and subsequent loss of  $\text{CO}_2$ , the alkalinity of the blood can rise and produce a pH of 7.40. Considerable acidosis occurs in precomatose stages and during diabetic coma (failure of regulation). In these cases pH values of 7.2 and less can be encountered. Low pH values are also found in kidney diseases, especially in uremic patients. Various heart diseases will give low pH values, even during rest.

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- <sup>16</sup> D. D. VAN SLIKE AND J. SENDROY JR.: *J. Biol. Chem.* **79**, 781, 1928.



$$0.10 + \log \frac{40.73 - 0.07 \times 40}{0.07 \times 40}$$

$$0.10 + \log \frac{40.73 - 2.08}{2.08}$$

$$0.10 + \log \frac{44.05}{2.08}$$

$$0.10 + \log 16.43$$

$$0.10 + 1.2150 = 7.3150$$

### *Determination of the pH in Arterial Plasma*

When arterial blood (serum or plasma) is used for the analysis, the procedure is much simpler as only the total  $\text{CO}_2$  of the plasma, drawn under the above described precautions has to be determined, the  $p_{\text{CO}_2}$  is assumed to be constant (40 mm Hg). The  $\text{CO}_2$  content is determined by analysis and the pH can be read off directly from the van Slyke nomogram<sup>14</sup> (fig. 18). The point for the  $\text{CO}_2$  content which is found on the left line of the nomogram is connected by a straight line with the constant  $\text{CO}_2$  tension (40 mm Hg) (right line) and at the point of intersection of the line with the center line the pH can be read. This value is called the reduced pH.

### *Example*

In the arterial plasma the determined volume per cent of combined and free  $\text{CO}_2$  are 56.5 volume per cent. From the nomogram reading the tension of 40 mm corresponds to a pH of 7.40.

The pH may also be calculated according to the Hasselbach formula (equation 3. See p. 73)

$$\text{pH} = 0.10 + \log \frac{\text{vol. \% CO}_2 - 0.007 p}{0.007 p}$$

$$0.10 + \log \frac{56.5 - 40 \times 0.007}{40 \times 0.007}$$

$$0.10 + \log \frac{56.5 - 2.08}{2.08}$$

$$0.10 + \log \frac{53.82}{2.08}$$

$$0.10 + \log 20.08$$

$$0.10 + 1.30 = 7.4$$

Only serum or plasma can be used in this method. In whole blood the hydrogen ion concentration and the  $\text{CO}_2$  combining power are dependent upon the oxygen saturation of the hemoglobin, the oxy hemoglobin acts as a weak acid. Therefore the pH of the whole blood cannot be determined by these methods.

The pH is normally kept constant by the entire organism. It is maintained by the process of ventilation (decreased or increased exhalation of  $\text{CO}_2$  by the lung) and by the neutralizing action of basic metabolism products ( $\text{NH}_3$ ) and excretion through the intestinal tract and the kidneys (partly also in perspiration).

The pH of the arterial plasma of a normal resting person is between 7.38 and 7.40. During voluntary hyperventilation and subsequent loss of  $\text{CO}_2$  the alkalinity of the blood can rise and produce a pH of 7.46. Considerable acidosis occurs in precomatose stages and during diabetic coma (failure of regulation). In these cases pH values of 7.2 and less can be encountered. Low pH values are also found in kidney diseases especially in uremic patients. Various heart diseases will give low pH values, even during rest.

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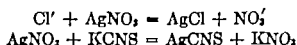
## Chapter III

# Determination of the Anions

### DETERMINATION OF THE CHLORIDES<sup>1</sup>

*Principle of the method* A known amount of a reagent consisting of a solution of silver nitrate, ceric nitrate and ferric ammonium sulfate in nitric acid added to blood (serum) destroys its proteins and simultaneously precipitates the chlorides as AgCl. The excess of AgNO<sub>3</sub> is titrated with potassium thiocyanate solution, the ferric ammonium sulfate serving as indicator.

#### Equation



#### Reagents

(1) Silver-cerium reagent Into a 100 ml volumetric flask are placed 10 ml of a N/10 AgNO<sub>3</sub> solution, 20 Gm. of pulverized ceric ammonium nitrate, 40 ml of concentrated HNO<sub>3</sub> and 15 ml of a cold saturated ferric ammonium sulfate solution. After the cerium salt has been dissolved, the flask is made up to the mark with water.

(2) Approximately 60 per cent glucose solution or a 50 per cent aqueous solution of glycerin (no mold formation)

(3) N/100 potassium thiocyanate solution prepared from N/10 thiocyanate solution

*Preparation of the N/10 AgNO<sub>3</sub> solution* 10.987 Gm of chemically pure silver nitrate are dissolved in halogen free dilute nitric acid and made up to 1 liter. An alternate way for the preparation of N/10 AgNO<sub>3</sub> is to dissolve 10.788 Gm pure metallic silver in halogen free nitric acid. After the metal has dissolved the nitrous acid must be destroyed by boiling. The solution is made up to 1 liter with water and must be kept in a dark bottle protected from light.

*Preparation of the N/10 potassium thiocyanate solution* Since KCNS is hygroscopic the exact solution cannot be made up by weighing of the salt. Eleven Gm. of potassium thiocyanate (somewhat more than the theoretical amount) and 10 ml of 40% formalin

are dissolved in 1000 ml of water (the formalin keeps the KCNS from being decomposed no ammonium thiocyanate must be used here) To 10 ml of the N/10  $\text{AgNO}_3$  solution (prepared as described above) a few drops of ferric ammonium sulfate are added and titrated with thiocyanate to a pale orange If 10 ml of silver nitrate solution use up 9.85 ml. of KCNS till the appearance of the orange color 98.5 ml of this KCNS solution and 10 ml of formalin must be made up to 1 liter to obtain a N/100 thiocyanate solution

### *Procedure*

Hagedorn-Jensen tubes (see blood sugar determination) 30 mm diam 100 mm height) are filled with approximately 1 ml of distilled water and 0.1 ml of blood (serum or spinal fluid) is added carefully from an exactly calibrated pipet The pipet is rinsed several times by sucking the mixture back and forth Two ml of the  $\text{AgNO}_3$ -cerium reagent (1) is added, the tubes are placed into a cold water bath and slowly heated to boiling They are left in the boiling water bath for 5-10 minutes (if no cerium salt is available, it is omitted in the reagent (1) and 0.5 ml of a saturated potassium permanganate solution is substituted The rest of the procedure boiling reducing is the same) After the addition of 0.5 ml of glucose solution (2) or glycerin solution the excess ceric ammonium nitrate (or  $\text{KMnO}_4$ ) is destroyed by heating the tubes in a hot water bath (with the flame removed) for 2 minutes The solution is allowed to cool to room temperature and is titrated with potassium thiocyanate from a microburette till the appearance of an orange color

For the blank determination 2 ml of solution (1) is used and treated like the sample.

### *Calculation*

Amount of potassium thiocyanate used up by the blank minus amount of potassium thiocyanate used up by the sample multiplied by 585 gives the chloride content expressed as NaCl in mg per cent multiplied by 355 gives the chloride content expressed as Cl in mg per cent.

With this method a great number of determinations may be carried out at the same time

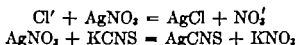
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### *Calculation*

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With this method a great number of determinations may be carried out at the same time.

*Example*

KCNS used by the blank	1.90 ml
KCNS used by the sample	1.01 ml
difference	0.98 ml

The sodium chloride content of the sample is  $0.98 \times 585 = 573.3$  mg per cent calculated as NaCl, or  $355 \times 0.98 = 347.90$  mg per cent calculated as Cl (see table 5)

The normal sodium chloride content of serum is 570–625 mg. per cent, that of whole blood is 450–510 mg per cent. The blood sample for the determination must be taken without tourniquet since incorrect values are obtained when chloride ions migrate from the serum into the red cells.

A rise of chlorides in serum occurs during anemia, in nephritis, essential hypertension, cardiac decompensation, urine retention, hyperventilation and during excessive intake of NaCl.

Low chloride values are encountered in excessive vomiting, nephritis and urinary retention accompanied by vomiting and diarrhea, in cases of edema, sublimate poisoning, diabetes mellitus, infectious diseases, emphysema, Addison's disease. In different cases of diabetes insipidus either high or low values are found, depending upon the nature of the disease. Low values are also found, if over a long period of time the diet is either low in or completely free from chlorides.

The NaCl content of spinal fluid varies between 720–750 mg per cent (expressed as NaCl) in adults and between 650–750 mg per cent in children. It rises in some cases of nephritis and falls in all meningitis diseases, especially low in tb-meningitis. The latter is a very important diagnostic factor.

BROMINE DETERMINATION<sup>2</sup>*Bromine Determination Using the Digestion Method*

*Principle of the method* The organic substance is destroyed with chromic acid-sulfuric acid in a closed system. The liberated halogens, chlorine and bromine combine with NaOH in a receiver. The bromide and hypobromite are oxidized to bromate with hypochlorous acid according to the formula

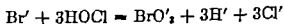


TABLE 5—Ml. N/100 thiocyanate corresponds to mg% NaCl. Ml. N/100 thiocyanate corresponds to mg% Cl

	00	001	002	003	004	005	006	007	008	009
0 0		3 55	7 10	10 65	14 20	17 75	21 30	24 85	28 40	31 95
0 0		5 85	11 70	17 55	23 40	29 25	35 10	40 95	46 80	52 65
0 1	35 50	39 05	42 60	46 15	49 70	53 25	56 80	60 35	63 90	67 45
0 1	58 50	64 35	70 20	76 05	81 90	87 75	93 60	99 45	105 30	111 15
0 2	71 00	74 55	78 10	81 65	85 20	88 75	92 30	95 85	99 40	102 95
0 2	117 00	122 85	128 70	134 55	140 40	146 25	152 10	157 95	163 80	169 65
0 3	106 50	110 05	113 60	117 15	120 70	124 25	127 80	131 35	134 90	138 45
0 3	175 50	181 35	187 20	193 05	198 90	204 75	210 60	216 45	222 30	228 15
0 4	142 00	145 55	149 10	152 65	156 20	159 75	163 30	166 85	170 40	173 95
0 4	234 00	239 85	245 70	251 55	257 40	263 25	269 10	274 95	280 80	286 65
0 5	177 55	181 05	184 60	188 15	191 70	195 25	198 80	202 35	205 90	209 45
0 5	292 50	298 35	304 20	310 05	315 90	321 75	327 60	333 45	339 30	345 15
0 6	213 00	216 55	220 10	223 65	227 20	230 75	234 30	237 85	241 40	244 95
0 6	351 00	356 85	362 70	368 55	374 40	380 25	386 10	391 95	397 80	403 65
0 7	248 50	252 05	255 60	259 15	262 70	266 25	269 80	273 35	276 90	280 45
0 7	409 50	415 35	421 20	427 05	432 90	438 75	444 60	450 45	456 30	462 15
0 8	284 00	287 55	291 10	294 65	298 20	301 75	305 30	308 85	312 40	315 95
0 8	468 00	473 85	479 70	485 55	491 40	497 25	503 10	508 95	514 80	520 65
0 9	319 50	323 05	326 60	330 15	333 70	337 25	340 80	344 35	347 90	351 45
0 9	538 50	542 35	546 20	549 05	552 90	556 75	560 60	564 45	568 30	572 15
1 0	355 00	358 55	362 10	365 65	369 20	372 75	376 30	379 85	383 40	386 95
1 0	585 00	590 85	596 70	602 55	608 40	614 25	620 10	625 95	631 80	637 65
1 1	390 50	394 05	397 60	401 15	404 70	408 25	411 80	415 35	418 90	422 45
1 1	643 50	649 35	655 20	661 05	666 90	672 75	678 60	684 45	690 30	696 15
1 2	426 00	429 55	433 10	436 65	440 20	443 75	447 30	450 85	454 40	457 95
1 2	702 00	707 85	713 70	719 55	725 40	731 25	737 10	742 95	748 80	754 65
1 3	461 50	465 05	468 60	472 15	475 70	479 25	482 80	486 35	489 90	493 45
1 3	760 50	766 35	772 20	778 05	783 90	789 75	795 60	801 45	807 30	813 15
1 4	497 00	500 55	504 10	507 65	511 20	514 75	518 30	521 85	525 40	528 95
1 4	819 00	824 85	830 70	836 55	842 40	848 25	854 10	859 95	865 80	871 65
1 5	532 50	536 05	539 60	543 15	546 70	550 25	553 80	557 35	560 90	564 45
1 5	877 50	883 35	889 20	895 05	900 90	906 75	912 60	918 45	924 30	930 15
1 6	563 00	571 55	575 10	578 65	582 20	585 75	589 30	592 85	596 40	599 95
1 6	938 00	941 85	947 70	953 55	959 40	965 25	971 10	976 95	982 80	988 65
1 7	003 50	007 05	010 60	014 15	017 70	021 25	024 80	028 35	031 90	035 45
1 7	994 50	1000 35	1006 20	1012 05	1017 90	1023 75	1029 60	1035 45	1041 30	1047 15
1 8	639 00	642 55	646 10	649 65	653 20	656 75	660 30	663 85	667 40	670 95
1 8	1033 00	1038 85	1044 70	1050 55	1056 40	1062 25	1068 10	1073 95	1079 80	1085 65
1 9	074 50	078 05	081 60	085 15	088 70	092 25	095 80	099 35	102 90	106 45
1 9	1111 50	1117 35	1123 20	1129 05	1134 90	1140 75	1146 60	1152 45	1158 30	1164 15



The excess of hypochloric acid is destroyed by sodium formate and the bromate determined iodometrically

### Reagents

(1) chromic acid 250 Gm. of chromic acid is dissolved in 150 ml. of redistilled water and a current of air, washed with NaOH, is drawn through the solution for 2 hours. One ml. of this solution contains approx. 1 Gm. of chromium trioxide

(2) silver sulfate-sulfuric acid 20 Gm. of silver sulfate is dissolved in 1 liter of concentrated sulfuric acid in a 2 liter Kjeldahl flask, 3 ml. of chromic acid solution (1) is added a thermometer is inserted and the flask is heated to 120-130° C and aerated as described under 1)

(3) crystalline boric acid

(4) sodium chloride, crystalline and a saturated solution of NaCl. Since even NaCl of the purest grade will contain bromide the solution must be purified by saturation with HCl gas. The precipitate is filtered off, washed with a small amount of water, dried well and pulverized. Traces of HCl can be neglected

(5) hypochlorite solution this solution is used only to check the purity of the reagents and is not employed in the analysis. Seven grams of chlorine gas (from a tank or prepared from HCl and permanganate, not from calcium hypochlorite) is added to a solution of 12 Gm. of NaOH in 100 ml. of water. The titer of this solution remains unchanged for a period of 2 months

(6) N/2 NaOH prepared from purest grade NaOH

(7) sodium formate, 10 per cent sol.

(8) ammonium molybdate 5 per cent sol

(9) KI cryst

(10) 2 N HCl (approx 7 per cent prepared by diluting 20 ml. of conc HCl to 100 ml. with dist. water)

(11) N/100 or N/200 sodium thiosulfate solution

(12) starch solution 0.25 per cent

The formation of halogen vapors must be strictly avoided during the performance of the analysis. All reagents must be tested for bromine since even the purest material may contain traces of Br

### Apparatus

The apparatus is shown in figure 19. It consists mainly of a digestion chamber of 150-170 ml. capacity. The ground glass top

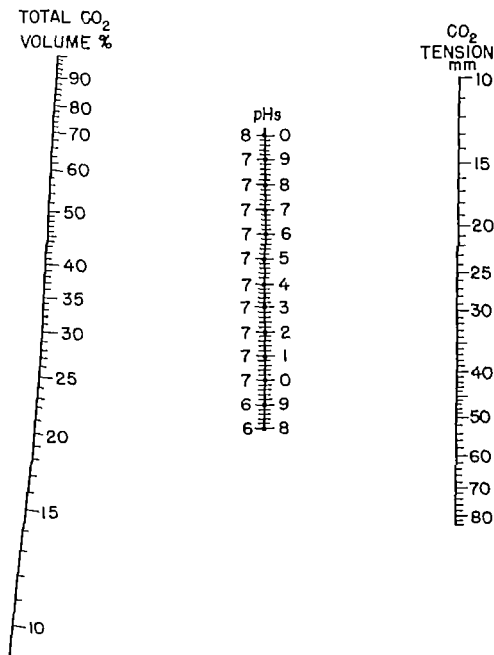


FIG. 18. Nomogram for the calculation of pH in serum (plasma) according to van Slyke (see p. 66).

is equipped with a dropping funnel (for the introduction of the sample) which is fused to a glass tube R<sub>1</sub> (1.5 ml. in diameter) with side arm. The stem of the funnel should reach down to about 5 cm.

above the surface of the liquid. In this arrangement each drop of sample falls directly to the bottom of the flask and is forced through

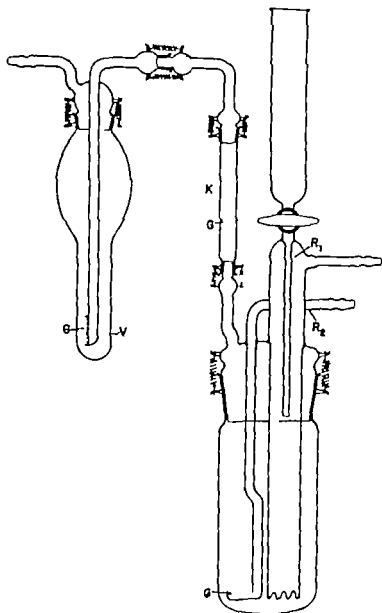


FIG 19 Apparatus for bromine determination according to Leipert.

the chromic acid layer. Any possibly present iodine is retained quantitatively (p 79) which would not be the case if the drops of sample would just touch the surface of the liquid. Also no water vapors can be formed. A second glass tube  $R_2$  carrying a

fritted glass plate G assures adequate ventilation. The chromic chloride is destroyed in a glass tube K of 9 cm. length and equipped with a fritted glass plate G. On this plate is put a 2 cm. deep layer of glass beads, moistened with sulfuric acid. This vessel is essential and must not be omitted from the apparatus. Chromic chloride is not formed when solid NaCl is heated with silver chromate-sulfuric acid, but its formation cannot be avoided when a NaCl solution is allowed to drop into the hot acid mixture with aeration. The rate of reaction is fastest in the flask K on account of the dense filter. In addition the warm current of air tends to concentrate the dilute sulfuric acid in the inserted vessel and therefore the solubility of the bromine is decreased. Thus no traces of bromine are left behind in the inserted vessel. The receiver V, carrying a fritted glass plate G to disperse the gas holds 10 ml. in the narrowed part. It is connected on one side to the digestion flask, on the other side to a water pump. The current of air, which is sucked through the apparatus passes first through a wash bottle filled with sodium hydroxide and is then divided by means of a Y tube into 2 parts. One part leads through rubber tubing carrying a pinch clamp to  $R_1$ , the other part in the same manner to  $R_2$ . The entire apparatus is held in place by a clamp attached at  $R_1$  above the ground glass piece. The glass tubings leading to and from the receiver are supported by rings. The digestion flask rests in a beaker filled with mineral oil up to the ground glass top so that the digestion process remains visible. The digestion flask, the inserted glass vessel and the glass tube leading to the receiver must be absolutely dry before the analysis. The ground glass top is easily dried by rinsing the inside part with acetone sucking air through and warming it carefully. Moisture in  $R_1$ ,  $R_2$  and the fritted glass plate can be neglected.

### *Digestion*

The digestion flask is filled with 100 ml. of silver sulfate-sulfuric acid. It is heated for a few minutes on the water bath. 8-10 ml. of chromic acid are added and the ground glass top is moistened with concentrated  $H_2SO_4$  to assure a tight seal. Then it is lowered into the beaker with mineral oil, which has been previously heated to 100° C. The fritted glass plate of the inserted vessel receives 0.2 ml. = 5-6 drops of sulfuric acid diluted 1:3. A 2 cm. deep layer of glass beads

is added, and it is connected with the digestion flask. The receiver is filled with 10 ml of  $N/2$  NaOH and it is connected with the pump on one side and with the apparatus on the other side.  $R_1$  and  $R_2$  are connected to the wash bottle as described above. Both pinch clamps are open. A current of air is sucked through the apparatus with the speed of 2 bubbles per second in the wash bottle. The air will take the path of least resistance, i.e., through  $R_1$ . Five ml of blood are pipetted into the funnel and hemolyzed with water. The speed of addition of the blood is so regulated that the liberated  $CO_2$  can easily be removed by the pump without rise of pressure in the system. 20 minutes being the average time for the addition of 5 ml of blood. The funnel is washed once with water, the airflow is reduced and the funnel again washed with 5 ml of saturated sodium chloride solution, diluted 1:10. In this procedure chromic chloride can be recognized as a yellow hue appearing on the fritted glass plate in the inserted glass vessel. If during the addition of the NaCl the current of air should be too strong, more chromic chloride is formed, but even in cases where the glass beads assume a yellow color no trace of chromic chloride reaches the receiver. After the NaCl has been added, air is passed through the apparatus at its original speed and the current of air flowing through  $R_1$  is slowed down so that the glass plate near  $R_2$  is well ventilated, whereas at  $R_1$  only one large bubble per 3 seconds is visible. An aeration time of  $1\frac{1}{2}$  hours from the beginning of the determination is sufficient to release all the liberated bromine. The oil bath temperature is raised slowly from  $100^\circ C$  to  $130^\circ C$  and is maintained at  $130^\circ C$  during the last half hour. After this time the inserted vessel and receiver are separated from the digestion flask, leaving the pump turned on. The inserted vessel is then separated and finally the receiver is disconnected from the pump.

#### *Bromine determination in the absorption liquid*

Since relatively large amounts of sodium chloride are used, the purified salt must be tested carefully. Five grams of NaCl are dissolved in 25 ml of water. 0.5 ml of hypochlorite solution and 1 Gm. of boric acid are added and it is heated for ten minutes with shaking in a boiling water bath to dissolve the boric acid. Then 2 ml of formate solution (7) are added, it is heated for 5 minutes over a

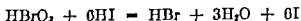
flame and after cooling a few crystals of KI starch solution, a drop of ammonium molybdate (8) and 5 ml. of HCl (10) are added. The liberated iodine is titrated. The appearance of a blue color before acidification indicates incomplete destruction of the hypochlorite and will occur when the original solution was too alkaline i.e. when not enough boric acid has been used. In such a case the solution is titrated with thiosulfate till the color disappears. Now acid is added and the titration performed as usual. The purified NaCl still contains traces of bromine, not more than 2-5 of bromine per 5 Gm. of NaCl but since only 0.5-1 Gm. of NaCl is used for the analysis these amounts of bromine fall within the margin of error of the method.

No hypochlorite has to be added to the receiver since the chloride content of the sample and of the funnel washings furnish a large excess of hypochlorite. The oxidation is performed directly in the receiver after the ground glass part and the glass tubing have been rinsed carefully. One gram of boric acid and 0.5 Gm. of NaCl are added to the solution in the receiver and the flask is placed into a boiling water bath for ten minutes. Then the contents of the flask are transferred into a 50 ml Erlenmeyer flask reduced with the formate solution (7) and treated as described above. It is acidified with 3 ml. of HCl (10). A microburette according to Bang is used for the titration.

### Calculation

The bromine in the receiver is oxidized to bromic acid by the hypochloric acid. As mentioned above the excess hypochloric acid which would interfere with the iodometric determination is reduced with sodium formate whereas the bromate is not affected by this reagent.

### Equation



shows that one atom of bromine is equivalent to 6 atoms of iodine. Consequently 1 ml of N/100 sodium thiosulfate solution corresponds to 0.1333 mg of bromine or 1 ml of N/200 sodium thiosulfate corresponds to 0.0666 mg of bromine.

*Example*

5 ml of blood after digestion	
require	2.45 ml N/200 thiosulfate
reagent blank	0.15 ml N/200 thiosulfate
difference	2.30 ml N/200 thiosulfate

$2.30 \times 0.0066 = 0.15318$  mg This figure represents the bromine content of 5 ml of blood. Multiplied by 20 the bromine content of 100 ml of blood is obtained i.e., the above sample contains 3.0636 mg per cent bromine.

The bromine content of blood in normal persons as determined by this method varies between 2.8 and 3.3 mg per cent. It rises after bromide medication.

#### METHOD FOR THE DETERMINATION OF BROMINE IN 0.1 ML. OF BLOOD WITHOUT DIGESTION<sup>2</sup>

*Principle of the method* The general procedure consists of deproteinization of the blood with zinc sulfate and sodium hydroxide, the oxidation of the bromide in the filtrate to bromate, and the iodometric titration of the bromate.

*Reagents*

- (1) 0.45 per cent zinc sulfate solution
- (2) 0.1 N sodium hydroxide solution
- (3) 2N hydrochloric acid
- (4) Calcium hypochlorite solution. A 15 per cent solution of

$\begin{array}{c} \text{OCl} \\ \diagdown \\ \text{Ca} \\ \diagup \\ \text{Cl} \end{array}$ 
 is filtered and the filtrate is diluted with water 1:3

- (5) calcium carbonate
- (6) 20 per cent sodium formate solution
- (7) 10 per cent potassium iodide solution
- (8) starch solution
- (9) 0.0004 N sodium thiosulfate solution

*Procedure*

The 0.1 ml. of blood is discharged directly from the pipet in which it is drawn into 0.5 ml. of 0.45 per cent zinc sulfate solution in a test

tube, 0.5 ml. of 0.1 N sodium hydroxide solution is then added and the tube is shaken, placed in a boiling water bath for three minutes, and then cooled. The fluid is next filtered and 5 ml. of the filtrate are placed into a second tube. 0.3 ml. of 2N hydrochloric acid, 1 ml. of calcium hypochlorite solution and a pinch of calcium carbonate are added in the order described. The carbonate should be slightly in excess, as indicated by its persistence as a precipitate. The tube is then placed in a boiling water bath for eight minutes after which 0.4 ml. of 20 per cent sodium formate solution is added and the tube is heated for eight minutes more. The tube is then removed from the bath and cooled. There are added in succession 3 ml. of 2N hydrochloric acid and 1 ml. of 10 per cent potassium iodide solution. The tube is shaken and 0.5 ml. of starch solution is added. The contents are then titrated with 0.0004 N sodium thiosulfate solution.

### *Calculation*

A blank determination is made whenever new reagents are prepared, the blank ordinarily ranges between 1.0 and 1.7 ml. of thiosulfate and should be subtracted from the volume of thiosulfate used in the analysis.

Each ml. of 0.0004 N thiosulfate corresponds to 7.6 mg. per 100 ml. of Br or 9.5 mg. per 100 ml. of sodium bromide in the blood being tested.

## IODINE DETERMINATION

### *Determination of Minute Amounts of Iodine in Blood<sup>1</sup>*

*Principle of the method.* The organic substance is destroyed with chromic acid, sulfuric acid in the presence of ceric sulfate as a catalyst. Iodine is quantitatively converted into non-volatile iodic acid which is then reduced with arsenious acid and the liberated iodine is distilled in vacuo into a receiving flask, containing alkali. It is again oxidized to iodic acid and determined volumetrically.

### *Reagents*

The reagents must be of the purest grade and completely free from iodine.

(1) sulfuric acid conc.

(2) chromic acid. 2.0 Gm. of chromic acid are dissolved in 150 ml.



of redistilled water. One ml. of this solution contains approximately 1 Gm. of chromium trioxide.

(3) ceric sulfate crystals

(4) arsenious acid

(a) arsenic trioxide

(b) saturated potassium carbonate solution purified according to Fellenberg (1000 Gm. of  $K_2CO_3$  are shaken 10 times with fresh 80 per cent ethyl alcohol (1000 ml.) After the first few shakings the alcohol can easily be decanted, later it has to be separated in a separatory funnel. An 85-90 per cent aqueous potassium carbonate solution is thus obtained.)

For the preparation of the arsenious acid 20 ml. of the potassium carbonate solution are diluted 10 times with water heated to boiling and 50 Gm. of arsenic trioxide are added with stirring. After cooling a small amount of undissolved arsenic trioxide is filtered off.

The arsenious acid solution may also be prepared as follows: a saturated solution of arsenic trioxide in 20 per cent NaOH (reagent 5) is made up and after cooling filtered through glass wool. Trevor and Fashena<sup>1</sup> have used phosphorous acid instead of arsenic trioxide with good results. The reagent is made up as follows: 'A c.p. grade of phosphorous acid is melted and a minimum amount of water added to prevent solidification. This was steam-distilled a constant volume being maintained until the distillate was free of chloride.'

(5) NaOH 20 per cent solution prepared from pure NaOH

(6) sulfurous acid

(7) aqueous methyl orange solution

(8) freshly prepared aqueous bromine solution

*Purification of the sulfuric acid* If a purification of the sulfuric acid should be necessary a 2000 ml. Kjeldahl flask is filled with 1 liter of sulfuric acid and 0.5 ml. of the chromic acid solution. The mixture is heated in an oil bath to  $120^\circ C$ . The excess chromic acid is reduced by the addition of traces of arsenic trioxide and a stream of air washed with alkali is passed through for 1 hour.

*Apparatus* For the distillation an apparatus as shown in figure 20 is used.

A represents a digestion flask (500 ml. Kjeldahl flask with ground glass top). The inner tube of the condenser carrying at its end a

fritted glass plate extends into the receiver  $V_1$  (4 cm wide at the neck)  $V_2$  is a similar smaller receiver. The lower part of the receiver holds each 10 ml of liquid. The arsenous acid is added through a dropping funnel.  $T$  is a T tube which is connected on one side with the distilling flask and on the other side with the steam generator  $D$  which contains slightly alkaline ( $\text{NaOH}$ ) water. The third arm of the  $T$  leads to the outside and may be connected to a suction pump.  $H_1$ ,  $H_2$ ,  $H_3$  and  $H_4$  are pinch clamps which serve to regulate the vacuum and the steam. In order to regulate the pressure

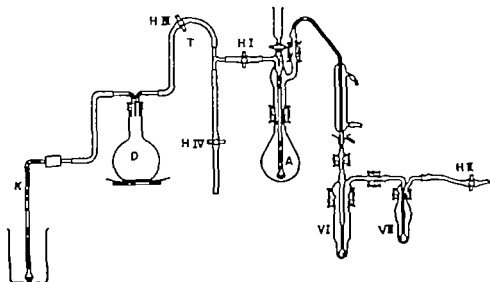


FIG. 20 Apparatus for iodine determination according to Lelpert.

a piece of glass tubing  $K$  equipped with a bulb with holes at its lower end and dipping into a beaker with water is connected to the steam generator

### Procedure

Into the digestion flask  $A$  are placed a trace of ceric sulfate 10-20 ml. of whole blood or serum and a suitable amount of chromic acid (10 ml of blood require approximately 18 ml. of chromic acid for complete digestion). The flask is placed on a Babo funnel (large metal funnel lined with asbestos paper) and the reaction is allowed to start. Then 100-150 ml of concentrated sulfuric acid are added slowly and with shaking. The first part of the acid must be added

with caution. The digestion mixture heats up considerably by itself and part of the excess water will evaporate. Sulfuric acid is added when the reaction slows down. The speed of reaction may be controlled by outside cooling. When large amounts of chromic acid have been used, a precipitate of chromium trioxide will appear when the concentration of sulfuric acid increases. This precipitate can be neglected and the rest of the sulfuric acid is added in larger portions. During this time chlorine (gas) derived from the sodium chloride content of the blood is liberated. The reaction is completed by heating for a short time on the Babo funnel. This heating period serves to remove the chlorine entirely and to destroy most of the chromic acid ( $O_2$  liberation). The digestion mixture turns to a greenish yellow color. The entire procedure should last approximately twenty minutes.

The flask is allowed to cool, first by itself, then by outside cooling, it is then carefully diluted with water, boiled again to remove chromyl chloride, and again diluted with water to prevent the development of  $SO_2$  vapors during distillation. Now the flask is connected to the apparatus. The ground glass parts can be sealed with a few drops of water. The steam generator is heated, the clamp  $H_1$  is closed, clamps  $H_2$  and  $H_4$  are open and the steam escapes through  $K$  into the open air. The receivers contain a few drops of 20 per cent  $NaOH$  and are filled with water up to the narrow part of the absorption flask. Now the apparatus is connected to the condenser and the suction pump.

### *Distillation*

Clamp  $H_2$  is opened to allow air to flow freely through the absorption vessels (receivers). It is not necessary to evacuate the apparatus completely, since a vacuum often results in undesired foaming, 50-60 mm Hg are sufficient. As soon as the suction pump starts working, clamp  $H_4$  is closed and clamp  $H_1$  is opened to such a degree that no suction is observed at  $K$ . Under these conditions the digestion mixture will begin to boil rapidly, the vapors rise through the neck of the flask and reach the condenser. To prevent the vapors from going any further than the middle of the condenser, clamps  $H_3$  and  $H_5$  are shut a little. Rapid boiling occurs in the digestion flask

and occasional air bubbles pass through the receivers. Now the arsenious acid is added through the dropping funnel at the rate of 2-3 drops per second. If the vapors move any further past the middle of the condenser the current of steam must be slowed down. Small bubbles of  $\text{CO}_2$  are observed in the receivers originating from excess  $\text{K}_2\text{CO}_3$ . As soon as the chromic acid has been reduced the entire amount of iodine will distill over at once (150  $\gamma$  of iodine can be recognized by the transient yellow coloring of the condensation ring. Larger amounts of iodine appear as chunks. However these amounts will not normally occur in blood). When small amounts of iodine, such as normally found in blood, are to be determined the reducing agent is added until the contents of the flask turn blue-green. This point can easily be recognized even by an untrained observer. An excess of arsenious acid is not harmful because the iodine is liberated instantaneously from the digestion mixture. Prolonged distillation is therefore unnecessary.

Clamp  $\text{H}_1$  is now opened as far as the pressure permits and the condenser tube becomes gradually hot all the way to the bottom of the receiver. In this way any traces of iodine left in the condenser are vaporized and can combine with the  $\text{NaOH}$  in the receiver vessels. Clamp  $\text{H}_1$  is closed in order to allow rinsing of the condenser tube with the receiver fluid and it is opened again to allow the fluid to return to the flask. The condenser tube is finally washed out by the condensing water vapors. If large amounts of iodine are expected the condenser must be rinsed once more with fresh water. At the end of the distillation clamp  $\text{H}_4$  is opened to the outside air and clamp  $\text{H}_2$  is closed. Clamp  $\text{H}_3$  is closed and air is allowed to enter the apparatus slowly. Then both receivers are removed.

To clean the apparatus the free arm of T is connected to the pump, the digestion mixture is removed by suction and water is sucked through the apparatus through the condenser tube. Now the pump is disconnected, clamp  $\text{H}_4$  is closed and the apparatus is steamed out while the next analytical sample is prepared.

The iodine distillation may also be performed without vacuum in the apparatus shown in figure 21. Since iodine is volatile a steam distillation over a period of 10 minutes timed from the beginning of the boiling of the digestion mixture is sufficient to liberate and collect the iodine. During the first five minutes the end of the condenser

(2) approximately N/10 anhydrous sodium sulfite in proportion upon standing. It will precipitate. The receivers are filled with (1) small amounts of iodine are colorless when large amounts are to be determined.

- 3 31.6 Gm  $\text{KMnO}_4$  dissolve in 100 ml concentrated  $\text{H}_2\text{PO}_4$  (strong)
- 5  $\text{NaNO}_2$  10.4 per cent in 100 ml solution, 30 per cent
- 7 5 per cent KI (this solution is used at  $0^\circ\text{C}$ )

*Procedure* — Place the sample in a shallow water bath. Fill the pipet of a dry receiver with a permanent purple color. (1) 2 ml of (2) 10 per cent for small amount as necessary for 1 mg of iodine. Add the solution gently to the samples for two minutes (0.03 ml of (3) 0.24 ml.) of 85 per cent the samples containing 0.5 ml and 2 ml of (1) respectively. No decolorization of the purple occurs. After two minutes have elapsed add sodium (5) drop by drop directly into the solution until the dioxides and excess permanganate are reduced. Add solution in excess and thoroughly rotate the sides of the flask in order to reduce any manganese dioxide there. Finally after the solution has stood for 2 minutes drop of urea (6) to the 1 ml. volumes and 5 drops to the 5 ml. Rotate the solution thoroughly upon the walls of the flask but the sample to stand 4 minutes longer in the water bath the sample to room temperature ( $20-30^\circ\text{C}$ ) before titration.

*Titration* One drop (0.03 ml) of potassium iodide (7) is added to the samples, containing less than 10 to 20 mg. From 0.1 ml. of potassium iodide solution is then added to the samples containing up to 1 mg. of iodate iodine.

For the rest of the titration and the calculation see bromine method, p 84

The normal iodine content of blood varies between 12 and 14 per cent. The thyroid gland regulates the iodine level.

High values for iodine are found after iodide ingestion after thyroxin treatment and in Basedow's disease (30-50  $\gamma$  per cent). Low values are encountered in myxedema and cretinism (2-6  $\gamma$  per cent) and after total thyroidectomy.

#### MICRODETERMINATION OF TOTAL IODINE (AFTER IODINE SUPPLY)\*

This method can only be used when high iodine levels are to be expected (iodine medication), it is not suited for determinations in normal blood.

*Principle of the method.* The organic material is fused and ignited with potassium nitrate and sodium carbonate and the iodine is oxidized to iodate with potassium permanganate. The iodine is liberated from the iodate by the addition of iodide and titrated with sodium thiosulfate.

#### Reagents

(1) Two parts of finely pulverized dry, pure sodium carbonate and 1 part of finely pulverized dry, pure potassium nitrate (iodine free) are well mixed in a mortar.

(2) potassium permanganate solution saturated in the cold

(3) dilute  $\text{KMnO}_4$  solution prepared as follows. The saturated solution is diluted 1:10 with redistilled water. (This solution has to be made up fresh each time)

(4) 25 volume per cent sulfuric acid

(5) 0.7 per cent sodium nitrite solution

(6) 10 per cent urea solution

(7) 5 per cent KI solution

(8) 0.25 per cent starch solution

(9) N/200 sodium thiosulfate solution

#### Procedure

One gram of reagent (1) is weighed out into a small porcelain crucible and 1 ml. of blood is added slowly so that it is evenly absorbed by the salt mixture. The crucible is placed into an oven which is

(2) approximately N/10  $\text{Na}_2\text{SO}_3$  prepared by dissolving 1.3 Gm. anhydrous sodium sulfite in 100 ml of water. This solution decomposes upon standing. It will keep in the refrigerator for a week. The receivers are filled with 0.5 ml of (1) and 0.5 ml of (2) when small amounts of iodine are expected, with 2 ml of both reagents when large amounts are to be determined.

(3) 31.6 Gm  $\text{KMnO}_4$  dissolved in 1000 ml of water

(4) concentrated  $\text{H}_3\text{PO}_4$  (syrupy)

(5)  $\text{NaNO}_2$  10.4 per cent

(6) urea solution, 30 per cent

(7) 5 per cent KI (this solution should give no color with starch when cooled to  $0^\circ \text{C}$ )

### *Procedure*

Place the sample in a shallow water bath. Add potassium permanganate (3), from the pipet of a dropping bottle, directly into the solution until a permanent purple coloring results. Two drops (0.06 ml) are sufficient for small amounts of iodine, but as much as 8 drops may be necessary for 1 mg of iodine. After adding the permanganate, rotate the solution gently upon the sides of the flask and heat the samples for two minutes. Then add 2 drops (0.06 ml.) and 8 drops (0.24 ml) of 85 per cent phosphoric acid to the samples, containing 0.5 ml and 2 ml of potassium carbonate (1) respectively. No decolorization of the permanganate should occur. After two minutes have elapsed add sodium nitrite solution (5) drop by drop directly into the solution until all manganese dioxide and excess permanganate are reduced. Add 1 drop of nitrite solution in excess and thoroughly rotate the solution upon the sides of the flask in order to reduce any manganese dioxide particles there. Finally, after the solution has stood for 2 minutes, add 1 drop of urea (6) to the 1 ml volumes and 5 drops to the 5 ml volumes. Rotate the solution thoroughly upon the walls of the flask and permit the sample to stand 4 minutes longer in the water bath. Cool the sample to room temperature ( $20\text{--}30^\circ \text{C}$ ) before titration.

*Titration* One drop (0.03 ml) of potassium iodide (7) is added to the samples, containing less than 10 to 20 mg. From 0.2 to 1 ml. of potassium iodide solution is then added to the samples containing up to 1 mg. of iodate iodine.

For the rest of the titration and the calculation see bromine method, p 84

The normal iodine content of blood varies between 12 and 14 per cent. The thyroid gland regulates the iodine level.

High values for iodine are found after iodide ingestion, after thyroxin treatment and in Basedow's disease (30-50  $\gamma$  per cent). Low values are encountered in myxedema and cretinism (2-6  $\gamma$  per cent) and after total thyroidectomy.

#### MICRODETERMINATION OF TOTAL IODINE (AFTER IODINE SUPPLY)<sup>7</sup>

This method can only be used when high iodine levels are to be expected (iodine medication), it is not suited for determinations in normal blood.

*Principle of the method* The organic material is fused and ignited with potassium nitrate and sodium carbonate and the iodine is oxidized to iodate with potassium permanganate. The iodine is liberated from the iodate by the addition of iodide and titrated with sodium thiosulfate.

#### Reagents

- (1) Two parts of finely pulverized dry, pure sodium carbonate and 1 part of finely pulverized dry, pure potassium nitrate (iodine free) are well mixed in a mortar
- (2) potassium permanganate solution saturated in the cold
- (3) dilute  $\text{KMnO}_4$  solution prepared as follows. The saturated solution is diluted 1:10 with redistilled water. (This solution has to be made up fresh each time)
- (4) 20 volume per cent sulfuric acid
- (5) 0.7 per cent sodium nitrite solution
- (6) 10 per cent urea solution
- (7) 5 per cent KI solution
- (8) 0.20 per cent starch solution
- (9) N/200 sodium thiosulfate solution

#### Procedure

One gram of reagent (1) is weighed out into a small porcelain crucible and 1 ml. of blood is added slowly so that it is evenly absorbed by the salt mixture. The crucible is placed into an oven which is



gradually heated to 110° C over a period of 1-2 hours. After all the liquid has evaporated, the crucible is removed from the oven and heated over a flame till the contents have melted and the carbon particles have disappeared. The slightly green colored fused material is cooled and dissolved in 5 ml of hot water and transferred quantitatively by means of a pipet into a 100 ml Erlenmeyer flask, using about 15 ml of water to rinse the crucible. Ten to 15 drops of potassium permanganate solution (2) and several glass beads are added, it is well mixed by shaking and brought to a short boil (10 seconds). The flask is removed from the flame and 5 ml of sulfuric acid (4) are added to the hot solution. The first 1 or 2 ml must be added with caution since foaming will occur as a result of CO<sub>2</sub> and nitrous acid fumes. The remainder of the sulfuric acid is used to wash down the neck and sides of the flask. The contents must be clear and colorless, otherwise a few more drops of sulfuric acid must be added. The hot flask is put back on the flame and dilute KMnO<sub>4</sub> solution (3) is added till the pink color remains. The permanganate should not touch the sides of the Erlenmeyer flask, because manganese dioxide will form, which interferes with the reaction. The contents of the flask are boiled for 10-15 seconds and the potassium permanganate reduced by the addition of 0.1 ml of sodium nitrite solution (5). To remove excess nitrous acid 1 ml of 10 per cent urea solution (6) is added and it is boiled for one minute. The flask is cooled again, 1 ml of KI solution (7) is added and the liberated iodine is titrated with sodium thiosulfate (9) using starch (8) as indicator.

#### Calculation

1 ml of N/200 thiosulfate corresponds to  $\frac{0.0012692}{2 \times 6}$  Gm of iodine = 0.1057 mg iodine. The division by 6 is necessary, because the iodine was determined as iodate.

#### DETERMINATION OF PHOSPHORUS

The following phosphorus-containing fractions besides the total phosphorus can be determined in blood

(1) acid-soluble fraction

(a) inorganic phosphate

(b) organic phosphorus compounds which are not precipitated

by the usual protein precipitating agents and which contain combined  $\text{PO}_4$  (phosphoric acid esters)

(2) phospholipid fractions

(3) phosphates which are precipitated by the usual protein precipitating agents (phospho-proteins)

The determination of inorganic phosphorus is most frequently required in a clinical laboratory. The cellular elements of the blood contain mainly ionisable organic phosphorus compounds. Provided that the blood sample is neither hemolyzed or hemolytic an increase of organic bound acid soluble phosphorus occurs at the cost of the inorganic phosphorus during the first 2-3 hours after the blood has been drawn. (The inorganic phosphorus forms an organic compound. The values for inorganic phosphorus are then found to be smaller.) Later on the process is reversed and an increase in inorganic phosphorus occurs through rapid decomposition of the acid-soluble esterified phosphorus. The latter reaction takes place spontaneously in hemolyzed blood.

If the inorganic phosphorus or the acid-soluble esterified phosphorus is to be determined in whole blood, tissues or red cells, the proteins must be removed by acid precipitation and hemolysis must be carefully avoided (to prevent hydrolysis of the acid-soluble esters). For the determination of inorganic phosphorus in whole blood the protein free filtrate must not be heated and the analysis must be performed rapidly because the acid hydrolysis of the organic phosphorus takes place already at room temperature.

The blood must be centrifuged immediately after having been drawn and the serum must be separated from the cells. Only sera which are completely free from hemoglobin can be used for the determination. In the protein free filtrate of the serum (plasma) the esters are stable and may even be heated with dilute acid.

#### SEMIC-MICRO-DETERMINATION OF ACID-SOLUBLE PHOSPHORUS<sup>1</sup>

*Principle of the method* Phosphoric acid forms a complex phosphomolybdic acid with molybdic acid. A blue color results when this complex is reduced with  $\alpha$  aminonaphthol sulfonic acid. This blue color is compared with the color of a standard phosphate solution in the Dubouque or Hellige colorimeter.

*Determination of Inorganic Phosphorus**Reagents*

(1) 10 per cent trichloroacetic acid It is most important for this reagent to be very pure, any impurities will prevent the development of the color. Some preparations of trichloroacetic acid contain phosphorus, only the purest grade of acid should be used.

(2) ammonium molybdate 25 Gm. of ammonium molybdate are dissolved in 200 ml. of water (slight heating may be necessary). The molybdate solution is cooled and added with continuous stirring to 300 ml. of 10 N sulfuric acid (10 N sulfuric acid is prepared by adding 450 ml. of concentrated sulfuric acid to 1300 ml. of water).

(3) standard phosphate solution

(a) stock solution 0.3509 Gm. of pure monopotassium phosphate ( $\text{KH}_2\text{PO}_4$ ) and 80 Gm. of trichloroacetic acid are dissolved in water and the solution is made up to 1 liter with water.

(b) working standard

(a) for the Dubosque colorimeter 10 ml. of the stock solution corresponding to 0.8 mg. of phosphorus are added to 80 ml. of 10 per cent trichloroacetic acid solution in a 100 ml. volumetric flask. The mixture is made up to the mark with water.

(b) for the Hellige colorimeter 20 ml. of the stock solution corresponding to 1.6 mg. of phosphorus are filled into a 100 ml. volumetric flask and made up to the mark with 10 per cent trichloroacetic acid.

(4) 15 per cent sodium bisulfite solution ( $\text{NaHSO}_3$ ) Cloudy solutions must be discarded, fresh solutions should be allowed to stand for 2-3 days before filtering.

(5) sodium sulfite solution ( $\text{Na}_2\text{SO}_3 \times 7\text{H}_2\text{O}$ ) 200 Gm. are dissolved in 380 ml. of water and filtered.

(6)  $\alpha$ -aminonaphtholsulfonic acid The commercial preparations must be recrystallized. Dissolve 150 Gm. of  $\text{NaHSO}_3$  and 10 Gm. of  $\text{Na}_2\text{SO}_3$  in 100 ml. of water heated to  $90^\circ\text{C}$ . To this solution is added 15 Gm. of commercially available aminosulfonic acid, the hot solution is filtered and after cooling 10 ml. of concentrated  $\text{HCl}$  is added with stirring. The precipitate is filtered off by suction, washed with approximately 300 ml. of water, then with alcohol until the washings are colorless. The pure acid is air-dried in the dark.

(brown glass) pulverized and kept in a dark bottle. A 0.25 per cent solution of  $\alpha$ -ammonaphthol sulfonic acid is used for the determination. 0.5 Gm of dry powdered aminosulfonic acid is added to 195 ml of 15 per cent bisulfite solution ( $\text{NaHSO}_3$ ) and 5 ml of 20 per cent sodium sulfite solution ( $\text{Na}_2\text{SO}_3$ ) and the mixture is well shaken. (If the bisulfite solution is old and oxidized more than 5 ml. of sodium sulfite are required to dissolve the acid.) No excess sulfite should be used. The solution keeps for approximately 2 weeks, when kept cold and in the dark (icebox).

### Procedure

A test tube with ground glass top is filled with 8 ml. of trichloroacetic acid and 2 ml. of oxalated blood is added with shaking. After continued shaking it is filtered through an ash-free filter. Five ml. of filtrate (corresponding to 1 ml. of blood) 1 ml. of molybdate and 0.4 ml. of aminosulfonic acid reagent are pipetted into a 10 ml. volumetric flask, water added up to the mark and the contents well mixed. At the same time 5 ml. of the working standard phosphate solution ( $\alpha$  or  $\beta$ ) is pipetted into a 10 ml. volumetric flask, 1 ml. of molybdate and 0.4 ml. of aminosulfonic acid reagent are added, and made up with water to the mark. Maximal intensity of color develops after five minutes and remains constant over a prolonged period of time.

### Calculation

$$\frac{\text{reading of the standard} \times 0.04}{\text{reading of the sample}} = \text{mg. phosphorus in the sample.}$$

The result is multiplied by 100 to obtain the phosphorus content expressed in mg. per cent.

### Example

reading of the standard	20 mm.
reading of the sample	18 mm.
concentration of the standard	0.04 mg

$$\frac{20 \times 0.04 \times 100}{18} = 4.4 \text{ mg. per cent phosphorus content.}$$

If the Hellige colorimeter is used the standard is placed into the wedge of the instrument and the sample into the cup.

*Calculation*

$$0.03 \times \frac{100 - y}{100} = \text{mg. P} \quad y = \text{colorimeter reading.}$$

The result is multiplied by 100 to obtain the phosphorus content in mg per cent

*Determination of the Total Acid-Soluble Phosphorus (Inorganic Phosphorus and Phosphate Esters)*

*Reagents*

(1) 5 N  $\text{H}_2\text{SO}_4$  prepared from 10 N  $\text{H}_2\text{SO}_4$  by proper dilution with water (see determination of inorganic phosphorus, 2)

(2) 2.5 per cent aqueous ammonium molybdate solution, neutral  
2.5 Gm. of ammonium molybdate is dissolved in 500 ml of warm water and made up to 1 liter. The solution cannot be used when large amounts of precipitate (ammonium trimolybdate) are present

(3) standard phosphate solution

( $\alpha$ ) for the Dubosque colorimeter 20 ml of the stock solution (see determination of inorganic phosphorus reagent 3) is mixed with 80 ml. of 10 per cent trichloroacetic acid. Five ml of this solution correspond to 0.08 mg of phosphorus

( $\beta$ ) for the Hellige colorimeter 40 ml of the stock solution is mixed with 60 ml of 10 per cent trichloroacetic acid. Five ml. of this solution correspond to 0.16 mg of phosphorus.

(4) concentrated  $\text{HNO}_3$

(5) 2 per cent urea solution

other reagents, see determination of inorganic phosphorus.

*Procedure*

A micro-Kjeldahl flask (pyrex) is filled with 5 ml of the trichloroacetic acid filtrate another flask is filled with 5 ml of phosphorus standard ( $\alpha$  or  $\beta$ ). To each flask is added 5 ml of 5 N  $\text{H}_2\text{SO}_4$  and a few glass beads to prevent bumping. The flasks are heated for digestion over a microburner (see determination of non protein nitrogen p 137 figure 27) until sulfuric acid vapors appear. The flame is reduced and concentrated nitric acid is added drop by drop until the residue remains colorless. Two ml of 2 per cent urea solution is added (to combine with nitrous compounds if present) and

unknown samples are transferred into 50 ml volumetric flasks, 5 ml of molybdate and 2 ml of  $\alpha$ -aminonaphthol sulfonic acid reagent are added, made up to the mark with water and after ten minutes the samples are read in the colorimeter

#### Calculation

( $\alpha$ ) for the Dubosque colorimeter

$$\frac{\text{reading of the standard} \times 0.03 \times 100}{\text{reading of the unknown sample}} = \text{total acid}$$

soluble phosphorus (inorganic and organic) in mg per cent

( $\beta$ ) for the Hellige colorimeter

$$\frac{(100 - y) \times 0.16 \times 100}{100} \quad y = \text{colorimeter reading}$$

equals (organic and inorganic) phosphorus in mg per cent.

In order to obtain the acid-soluble combined organic phosphorus the value for organic phosphorus must be deducted from the above obtained result

### MICRO-DETERMINATION OF PHOSPHORUS AND ITS FRACTIONS<sup>9</sup>

*Principle of the method* Phosphoric acid and molybdic acid form a complex phosphomolybdic acid. The addition of stannous chloride ( $\text{SnCl}_2$ ) reduces only this phosphor complex to a blue molybdic oxide, the excess molybdic acid not being affected and remaining colorless. The phosphorus content of the sample is determined by comparison with a standard phosphate solution

#### Determination of Inorganic Phosphorus in Serum and Plasma

##### Reagents

(1) 14 per cent trichloroacetic acid pure, dissolved in redistilled water

(2) molybdic acid-sulfuric acid reagent

(a) 93 ml of concentrated  $\text{H}_2\text{SO}_4$  are poured into distilled water and after cooling it is made up to 1 liter

(b) 7.5 Gm of sodium molybdate (c p, reagent, free from potassium and ammonium) are dissolved in a 100 ml. volumetric flask in distilled water and made up to the mark with water. If the solution is turbid it is filtered through a fluted filter. For the anal

yeas 3 parts of solution (a) are mixed with one part of solution (b) The mixture keeps over a long period of time

(3) stannous chloride solution <sup>10,11</sup>

(a) stock solution In a 25 ml volumetric flask 10 Gm. of  $\text{SnCl}_2$  are dissolved in a small amount of concentrated  $\text{HCl}$  and made up to the mark with concentrated  $\text{HCl}$ . A few small pieces of phosphorus-free pure metallic tin are added to prevent autoxidation of the  $\text{SnCl}_2$  according to the formula  $\text{SnCl}_2 + \text{Sn} = 2\text{SnCl}_2$ . This solution will keep indefinitely, the metal is used up with time and has to be replaced approximately once a year

(b) the stock solution is diluted before use. The rate of dilution can be found by determining the concentration of the stock solution as follows into a 50 ml. measuring flask is placed 0.1 ml of  $\text{SnCl}_2$  and made up to the mark with distilled water. Test tubes of 2.5 cm. diameter are filled with 5 ml of  $\text{N}/200 \text{ KIO}_3$ ,  $\text{KI}$  is added, well mixed and 1 ml of diluted  $\text{SnCl}_2$  is added. After some seconds it is titrated with  $\text{N}/200$  sodium thiosulfate till yellow. Then the titration is continued with starch until the blue color disappears. In the same way two blank values without  $\text{SnCl}_2$  are titrated. The difference between blank and full value multiplied by  $0.5625 = \frac{(225000 \times \text{mol SnCl}_2)}{2 \times 200 \times 1000}$

gives  $\text{SnCl}_2$  in 1 ml. of the diluted  $\text{SnCl}_2$  solution. According to Kuttner and Liechtenstem the  $\text{SnCl}_2$  solution should contain 2 mg of  $\text{SnCl}_2$  per ml. The degree of dilution is calculated from the titration values

*Example*

blank value needs	5 ml of thiosulfate
full value needs	<u>2.8 ml of thiosulfate</u>
difference	2.2 ml

$$2.2 \times 0.5625 = 1.2375$$

1 ml of this solution thus contains 1.23 mg per cent  $\text{SnCl}_2$ . From the proportion  $\frac{0.1}{X} = \frac{1.23}{2}$  the amount of  $\text{SnCl}_2$  solution is found

which must be diluted to 50 ml. In this case  $(X = \frac{0.2}{1.23} = 0.16)$

0.16 ml. must be diluted to 50 ml. As the concentrated solution is

nearly constant, it should be controlled once in a time, respectively every time before use if the estimation is carried out only occasionally

The dilute  $\text{SnCl}_2$  solution will keep only for one day and must be prepared immediately before use

(4) standard phosphate solution

(a) stock solution 0.4380 Gm. of  $\text{KH}_2\text{PO}_4$  (pure and 70 Gm. of trichloroacetic acid (c.p. reagent) are dissolved in water in a 1000 ml volumetric flask and made up to the mark with water. One ml. of this dilution contains 0.1 mg. of phosphorus, corresponding to 0.225 mg. of  $\text{P}_2\text{O}_5$ .

(b) from this stock solution two working standards are prepared

( $\alpha$ ) in a 200 ml. volumetric flask 10 ml. of stock solution are mixed with 95 ml. of trichloroacetic acid (1) and made up to the mark with water. One ml. of this solution contains 0.005 mg. of phosphorus.

( $\beta$ ) in a 200 ml. volumetric flask 20 ml. of stock solution are mixed with 90 ml. of trichloroacetic acid and made up to the mark with water. One ml. of this solution contains 0.01 mg. of phosphorus. According to the amount of phosphorus expected in the sample either solution  $\alpha$ ) or solution  $\beta$ ) is used

(5)  $\text{N}/1 \text{ H}_2\text{SO}_4$

*Procedure*

From an exactly calibrated pipet 0.2 ml. of serum is discharged into a small test tube, containing 0.8 ml. of distilled water. The pipet is rinsed by repeated sucking up of the water. After the addition of 1 ml. of trichloroacetic acid (1) the tube is centrifuged, and 1.5 ml. of the clear supernatant is placed into the microcup of a Dubosque colorimeter with the aid of a regular pipet or a precision pipet (fig. 4). The other cup is filled with 1.5 ml. of standard solution. 1.2 ml. of sulfuric acid molybdic acid reagent is added to both cups and well mixed with a glass rod. Now 0.3 ml. of dilute  $\text{SnCl}_2$  solution is added to each cup. After fifteen seconds the maximum color development has been reached and the color will remain stable over a period of two hours. Thus a series of determinations may be made with the same standard solution. If the colors are too intense to afford proper reading both the unknown sample and the standard may be equally diluted with  $\text{N}/1 \text{ H}_2\text{SO}_4$ .

All the glassware used in this determination must be perfectly clean. Rinsing with redistilled water is recommended. The water



must have been redistilled (see appendix) and should not give a blue color with the reagents.

#### Calculation

$$\frac{\text{reading of the standard} \times 0.0075}{\text{reading of the sample}} = \text{mg. phosphorus in the sample.}$$

In order to obtain the phosphorus content expressed in mg per cent, the result is multiplied by  $\frac{10000}{15}$

$$\frac{\text{reading of the standard} \times 5}{\text{reading of the sample}} = \text{mg per cent P}$$

#### Example

reading of the standard	20 mm.
reading of the sample	18 mm.
concentration of the standard	0.0075 mg

$$\frac{20 \times 0.0075 \times 10000}{18 \times 15} = \frac{20 \times 5}{18} = 5.5 \text{ mg. per cent P}$$

If the Hellige colorimeter is used, 1 ml. of the supernatant (see above) is transferred to the cup. At the same time 10 ml. of the standard solution (3) is pipetted into a test tube. With stirring 0.8 ml. of molybdic acid-sulfuric acid reagent (2) and 0.2 ml. of the dilute  $\text{SnCl}_2$  solution are added to the sample. Similarly 8 ml. of the reagent (2) and 2 ml. of dilute  $\text{SnCl}_2$  solution are added to the tube containing the standard. The contents of this tube are transferred after 15 seconds into the wedge of the colorimeter and the colors compared. The ratio between reagent (2) and reagent (3) are identical in the unknown sample and in the standard despite the larger total amount of standard solution.

#### Calculation

$$\frac{100 - y}{100} \times 10 = \text{mg. per cent P} \quad y = \text{colorimeter reading}$$

#### Example

identical coloring obtained at a setting of 45

$$\text{mg. per cent P} = \frac{100 - 45}{100} \times 10 = \frac{55 \times 10}{100} = 5.5$$

*Determination of Inorganic Phosphorus in Whole Blood**Reagents*

(1) 7.77 per cent trichloroacetic acid 55.5 ml. of 14 per cent trichloroacetic acid is diluted to 100 ml. with redistilled water

All other reagents as indicated in the previous paragraph.

*Procedure*

With a perfectly dry and well calibrated capillary pipet 0.2 ml. of blood is drawn from the finger tip and delivered into a narrow test tube equipped with ground glass stopper. The tube contains 1.8 ml. of 7.77 per cent trichloroacetic acid solution. A small glass bead is placed into the tube, the tube is stoppered and vigorously shaken for a period of time, and finally centrifuged. Further determination and calculation is carried out according to directions for the determination of phosphorus in serum.

*Determination of Total Acid-soluble Phosphorus (Phosphate Esters)*

## METHOD (A)

*Reagents*

(1) titanium-sulfuric acid 0.1 Gm. titanium hydroxide is dissolved in 100 ml. of 10 N  $\text{H}_2\text{SO}_4$ . To obtain 10N  $\text{H}_2\text{SO}_4$ , 279 ml. of concentrated  $\text{H}_2\text{SO}_4$  is added to water and the solution made up to 1000 ml. with water

(2) hydrogen peroxide (30 per cent) pure

(3) molybdic acid reagent in a 100 ml. volumetric flask 1.9 Gm. of sodium molybdate (c.p. reagent) is dissolved in redistilled water and made up to the mark. Filter if cloudy

For all other reagents see determination of inorganic phosphorus.

*Procedure*

Serum or whole blood is deproteinized with trichloroacetic acid (see determination of inorganic phosphorus in serum and whole blood) and 1.5 ml. of filtrate is transferred to a pyrex digestion flask (volume of bulb approx 5 ml. length of flask 180-190 mm., diameter of neck 10 mm., bearing marks at 8 ml. and 10 ml.). This is followed by 1 ml. of titanium sulfuric acid, 0.2 ml. of hydrogen peroxide and a glass bead to assure steady boiling. The mixture is digested over a micro-burner in the setup as described for the determination of

non-protein nitrogen (p 137, fig 27) The digestion is interrupted with the appearance of sulfuric acid fumes. It is terminated when the red liquid (titanium sulfate with traces of  $H_2O_2$  forms a red pertitanic acid<sup>13 14</sup>) has turned colorless. If the digestion is incomplete a black residue remains. In this case 0.2 ml of fresh  $H_2O_2$  must be added and the digestion continued. The colorless residue is diluted with redistilled water which is allowed to run down along the neck of the flask. When traces of  $H_2O_2$  have adhered to the neck of the flask, the contents of the flask will turn red, and the digestion has to be continued until the red color disappears, since traces of  $H_2O_2$  will interfere with the determination. A blank determination is carried out along with the sample by digesting 3 ml of the dilute phosphorus standard ( $\alpha$ ) with titanium-sulfuric acid and  $H_2O_2$  under the same conditions.

Unknown and standard are now diluted to the 8 ml mark with redistilled water and 1 ml of molybdate reagent (3) and 1 ml. of dilute  $SnCl_2$  solution is added. They are well mixed with the aid of a capillary pipet and after five minutes the colors are compared in the colorimeter.

### Calculation

$$\frac{\text{reading of the standard} \times 0.015}{\text{reading of the sample}} = \text{total acid soluble phosphorus}$$

in mg, after multiplication with  $\frac{10000}{15}$  in mg per cent.

$$\frac{\text{reading of the standard} \times 10}{\text{reading of the sample}} = \text{mg. per cent P (total acid-soluble)}$$

The value for the esterified phosphorus is obtained by subtracting the value for inorganic phosphorus from the value for total acid soluble phosphorus.

If the Hellge colorimeter is used the method is the same as described above. Solution  $\beta$  serves as standard.

### Calculation

$$\frac{100 - y}{100} \times 20 = \text{mg. per cent P} \quad y = \text{colorimeter reading.}$$

## METHOD (B)

Hydrogen peroxide frequently contains phosphates as preservative. If no phosphorus-free  $H_2O_2$  is available the following method is used which precludes the use of  $H_2O_2$ .

*Reagents*

- (1) 10 N  $H_2SO_4$  prepared by diluting 279 ml. of concentrated sulfuric acid with water to 1000 ml
- (2) concentrated nitric acid (a.p. spec. gravity 1.4)
- (3) 2 per cent urea solution
- (4) 1.9 per cent clear aqueous sodium molybdate solution (without acid)

All other reagents as described for the determination of inorganic phosphorus

*Procedure*

Serum or whole blood is deproteinized with trichloroacetic acid (see determination of inorganic phosphorus in serum or whole blood) and 1.5 ml. of supernatant is transferred into a digestion flask (see method a). To this is added 1 ml. of  $H_2SO_4$  (1) and 0.4 ml. of  $HNO_3$  (2). The mixture is heated carefully over a microburner until sulfuric acid fumes appear. Should the sample not be colorless after cooling 0.4 ml. of  $HNO_3$  is again added and the digestion repeated. After cooling 1 ml. of urea solution (3) is added and the solution boiled for a few minutes in a water bath (to destroy nitrous acid). The determination is continued as described under (a).

*Lipid Phosphorus**Reagents*

- (1) alcohol-ether mixture. 3 parts of 95 per cent ethyl alcohol are mixed with 1 part of ether. Both reagents must be redistilled before use. For all other reagents see determination of total acid-soluble phosphorus

*Procedure*

Into a 10 ml. volumetric flask are placed approximately 6 ml. of the alcohol-ether mixture. Slowly and with shaking 0.2 ml. of whole blood (serum or plasma) are added from an exactly calibrated pipet and the flask is immersed in boiling water for 30 seconds to complete

the protein precipitation The sample is cooled to room temperature, made up to the mark with alcohol-ether and filtered into a dry test tube through quantitative filter paper (5 cm. diameter) Five ml. of the filtrate, corresponding to 0.1 ml. of the original material are transferred into a digestion flask (see determination of total acid soluble phosphorus) and the alcohol-ether is evaporated on a water bath To prevent explosions during evaporation over a free flame,

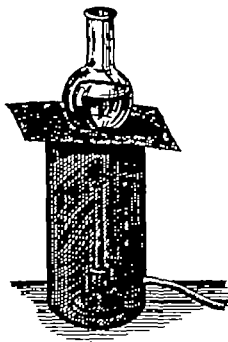


FIG 22 Safety burner for the evaporation of inflammable material

a variation of the Davy safety lamp is used (fig 22) When no more solvent can be detected (odor) 1 ml. of the titanium-sulfuric acid and 0.2 ml. of  $H_2O_2$  (i.e., the reagent described for method b) is added The remaining part of the procedure is performed as described for total acid soluble phosphorus. In this method 2 ml. of the standard phosphate solution are digested with titanium sulfuric acid and  $H_2O_2$  or  $H_2SO_4$  and  $HNO_3$  (method b)

#### Calculation

$$\text{Dubosque colorimeter} \frac{\text{reading of standard} \times 0.01}{\text{reading of the sample}} = \text{mg of lipid}$$

soluble phosphorus of the sample. By multiplication by 1000 the amount in mg per cent is obtained

*Example*

reading of the standard	20 mm.
reading of the unknown sample	22 mm
concentration of the standard	0.01 mg

$$\frac{20 \times 0.01 \times 1000}{22} = 9.09 \text{ mg. per cent lipid soluble phosphorus.}$$

*Calculation*

Hellige colorimeter (solution  $\beta$  is used)

$$\frac{100 - y}{100} \times 20 = \text{lipidphosphorus in mg. per cent}$$

$y$  = colorimeter reading

*Total Phosphorus*

*Reagents*

See inorganic and acid-soluble phosphorus.

*Procedure*

Into the digestion flask are placed 0.02 ml of whole blood or 0.05 ml of serum (plasma) (see digestion of acid-soluble phosphorus) and digested with 1 ml of acid (method a or b pp 97, 99) described above. In the same manner 3 ml of the phosphorus working standard solution ( $\alpha$  or  $\beta$  according to the colorimeter used) are digested. After the digestion the unknown sample and the standard are worked up as described above.

*Calculation*

$$\begin{aligned} \text{Dubosque colorimeter } \left[ \begin{aligned} &1) \frac{\text{reading of standard} \times 0.015 \times 100000}{\text{reading of sample} \times 20} \\ &= \frac{\text{reading of standard} \times 75}{\text{reading of sample}} \\ &= \text{total phosphorus in whole blood in mg. per cent} \end{aligned} \right. \\ \left[ \begin{aligned} &2) \frac{\text{reading of standard} \times 0.015 \times 100000}{\text{reading of sample} \times 50} \\ &= \frac{\text{reading of standard} \times 30}{\text{reading of sample}} \\ &= \text{total phosphorus in serum in mg. per cent} \end{aligned} \right. \end{aligned}$$

Hellige colorimeter [1] when working with 0.02 ml. of blood

$$\frac{100 - y}{100} \times \frac{0.03 \times 100000}{20} = \frac{100 - y}{100} \times 150$$

= total phosphorus in whole blood in mg. per cent

y = colorimeter reading

2) when working with 0.05 ml. of serum

$$\frac{100 - y}{100} \times \frac{0.03 \times 100000}{50} = \frac{100 - y}{100} \times 60$$

= mg. per cent total phosphorus.

The normal value for inorganic phosphorus in serum of adults is 3-4 mg per cent, in children 4-6 mg per cent. The phosphate ester content of the serum varies between 2 and 5 mg per cent in adults and between 4 and 7 mg per cent in children. The phosphate level is influenced by ultraviolet irradiation, thus it is highest in summer and lowest in winter.

Changes in the serum phosphate level can also be encountered during carbohydrate digestion, where an increase of phosphate esters (hexose phosphoric acid) at the expense of inorganic phosphorus is found (see glucose tolerance).

High serum levels are found in

(1) Hypervitaminosis (vitamin D) after administration of large doses of cod liver oil or Vigantol. Ultraviolet irradiation also causes a rise of serum phosphorus.

(2) Hypoparathyroidism. In decreased parathyroid function an increase in serum phosphorus is observed which is more or less proportional to a lowering of the serum calcium level.

(3) Nephritis. An increase in phosphorus is a sign of kidney insufficiency and is often found together with a decrease of alkali-reserve. Values of 8 mg per cent and above can be found in this disease. Similar changes occur in nephrosclerosis, in cystic kidneys, in pyonephrosis, hydronephrosis, and in tuberculosis of the kidney.

(4) During healing of bone fractures the phosphate level is increased. During this period values may vary between 5 and 7 mg per cent. Low serum levels are found in

(1) Rickets. In children values between 1 and 2 mg per cent have been encountered.





Hellige colorimeter (1) when working with 0.02 ml. of blood

$$\frac{100 - y}{100} \times \frac{0.03 \times 100000}{20} = \frac{100 - y}{100} \times 150$$

= total phosphorus in whole blood in mg. per cent

y = colorimeter reading

2) when working with 0.05 ml. of serum

$$\frac{100 - y}{100} \times \frac{0.03 \times 100000}{50} = \frac{100 - y}{100} \times 60$$

= mg. per cent total phosphorus.

The normal value for inorganic phosphorus in serum of adults is 3-4 mg per cent, in children 4-6 mg per cent. The phosphate ester content of the serum varies between 2 and 5 mg per cent in adults and between 4 and 7 mg per cent in children. The phosphate level is influenced by ultraviolet irradiation, thus it is highest in summer and lowest in winter.

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(1) Rickets. In children values between 1 and 2 mg per cent have been encountered.

- (2) Osteomalacia.
- (3) Hyperparathyroidism
- (4) Idiopathic steatorrhea celiac disease sprue of any kind.
- (5) During increased carbohydrate metabolism in hyperinsulinism and after insulin injections.

The phosphate content of spinal fluid varies between 1.20-2.10 mg per cent in adults and between 1.5-3.5 mg per cent in children. It depends upon the protein content. The level is higher in all

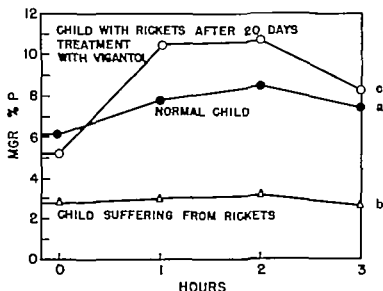


FIG 23 Phosphate tolerance curve (J Warkany)

degenerative and infectious processes of the central nervous system (tumors, tabes dorsalis, meningitis, etc.)

#### Phosphortolerance Tests<sup>14</sup>

The administration of 0.5 Gm per kg of body weight of secondary sodium phosphate to a normal person gradually results in a rise of the inorganic phosphate level of the serum. The maximum increase within three hours is approximately 3.5 mg. per cent (see curve a, fig 23). In a child suffering from rickets which has received the above mentioned dose of phosphate the maximum increase after the same period of time will be only 0.5 mg per cent (curve b fig 23). This abnormal behavior of patients suffering from rickets changes after prolonged administration of cod liver oil or Vigantol in such a

manner that the phosphate curve of the serum will be far above the normal curve (curve c, fig 23)

According to Klink<sup>13</sup> the phosphate fractions of whole blood and of serum are distributed as follows

	Total-P mg%	Lipid-P mg%	Phosphate-P mg%	Acid soluble P mg%
whole blood	37	10	8	24
serum (plasma)	13	3	7	3

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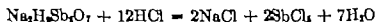
## Chapter IV

# Determination of the Kations

### DETERMINATION OF SODIUM<sup>1</sup>

*Principle of the method* The sodium is precipitated as insoluble salt with potassium pyro-antimonate and the 5 valent antimony is determined iodometrically

*Reaction equation*



### *Reagents*

(1) potassium pyro-antimonate In a pyrex Erlenmeyer flask 500 ml of distilled water is boiled approximately 5 Gm. of potassium pyro-antimonate ( $K_2H_9Sb_2O_7$ ) is added with stirring and the solution is boiled for exactly six minutes After rapid outside cooling with water, 15 ml. of 10 per cent KOH is added and the mixture is filtered through a double ash-free filter into a paraffinized bottle The solution should be allowed to stand at least twenty four hours before use

Preparation of a paraffinized bottle A piece of paraffin is placed into a clean, dry bottle and heated in an incubator to melt the paraffin Then the warm flask is rolled on a flat surface until it is cool.

(2) 20 per cent potassium iodide solution (freshly prepared each time)

(3) 0.25 per cent starch solution

(4) N/100 sodium thiosulfate solution

### *Procedure*

Blood is drawn without using a tourniquet and the serum is obtained by centrifugation

Into a 15 ml centrifuge tube 0.2 ml of serum or plasma is measured out and made up to 1 ml. with water All glassware (preferably

Jena or pyrex glass) should be brushed well with soap and water and rinsed with water. Cleaning solution is to be avoided. Now 5 ml. of the pyro-antimonate is added and the tube is cooled to  $10^{\circ}\text{C}$ . From a microburette 1.5 ml. of 95 per cent ethyl alcohol is added over a period of 30-40 seconds stirring constantly with a glass rod. The rod is washed with a few drops of distilled water and placed into a wide test tube (Hagedorn-Jensen tube made of pyrex glass), in which the titration will be performed later.

The cork-stoppered test tube is allowed to stand for  $\frac{1}{2}$  to 1 hour. Then it is centrifuged and the supernatant is carefully removed.

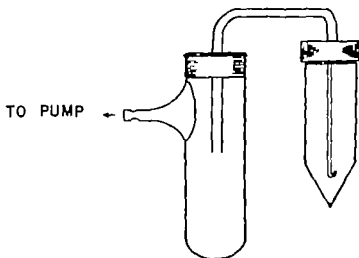


FIG 24 APPARATUS USED FOR SUCTION

without disturbing the precipitate (see fig 24). Five ml. of 30 per cent alcohol is added to the residue and centrifugation repeated. Then the supernatant is removed. Now the tube receives 5 ml. of concentrated HCl (chlorine-free) and the precipitate is dissolved by stirring with the same glass rod which has been used for the precipitation. The solution is transferred quantitatively into the Hagedorn-Jensen tube and the centrifuge tube is rinsed 3 times with 5 ml. of distilled water each. Two ml. of the freshly prepared 20 per cent potassium iodide solution are added and titration quickly performed with  $N/100 \text{ Na}_2\text{S}_2\text{O}_3$  till the appearance of a yellow color, then starch solution is added and titration continued till colorless.

*Calculation*

From the reaction equation it is evident that 1 atom of sodium corresponds to 2 atoms of iodine. Consequently 1 ml of N/100 thiosulfate solution corresponds to 0.115 mg of sodium.

$$\left( \frac{23}{2 \times 100 \times 1000} \right)$$

Multiplication of the result by 500 gives the sodium content in 100 ml of the sample. If 0.2 ml. of serum or plasma have been analyzed 1 ml. of N/100  $\text{Na}_2\text{S}_2\text{O}_3$  correspond to 57.5 per cent sodium.

*Example*

0.2 ml of serum require 5.80 ml of N/100  $\text{Na}_2\text{S}_2\text{O}_3$ . The sodium content of the serum is  $5.80 \times 57.5 = 333.5$  mg per cent.

Approximately 90 per cent of the total kation content of serum (plasma) consists of sodium. It is combined mostly with Cl to a smaller extent with  $\text{H}_2\text{CO}_3$ . Whole blood contains 170–200 mg per cent sodium, serum or plasma contains 320–350 mg per cent sodium. The sodium level of blood cannot easily be influenced. Very large oral doses of NaCl will change the level only slightly since the balance is soon regulated by water drawn into the blood stream from the tissues and by transferring excess salt to the organs.

The sodium level is changed in febrile infections especially in pneumonia, furthermore in diabetes insipidus. A rise in serum Na occurs during tuberculous pleura-exudate Cushing's disease and in cardio-renal disease. The sodium level is decreased in meningitis Addison's disease excessive sweating acidosis (diabetes mellitus). It is high during the menstrual period and during normal or pathological pregnancy. As a rule the sodium content is dependent upon the Cl content of serum (plasma).

The sodium content of spinal fluid is identical with that of plasma.

## DETERMINATION OF POTASSIUM

*Determination of Potassium in Serum and Plasma<sup>1,2</sup>*

*Principle of the method* Potassium is precipitated directly in the serum with Na-cobaltic nitrite as potassium sodium-cobaltic nitrite. The nitrite group of this double salt is oxidized by the addition of

a measured amount of a known ceric sulfate solution. The excess ceric sulfate is determined iodometrically.

#### *Reaction equation*



One potassium ion corresponds to 3NO<sub>2</sub> groups or 6 iodine atoms.

#### *Reagents*

##### (1) sodium cobaltic nitrite reagent

(a) for laboratories where daily potassium determinations are performed 5 Gm. of cobalt nitrate is dissolved in 100 ml. of redistilled water (see appendix). To this 2.5 ml. of glacial acetic acid and a solution of 23 Gm. of potassium free sodium nitrite in 35 ml. of redistilled water is added with ice-cooling. Nitrous oxide develops and air which has been washed with water is sucked through the solution until the gas evolution has stopped. The reagent must be kept in a well-closed bottle in the refrigerator, it will keep for 4 weeks at the most and must be filtered before each determination.

(b) for laboratories<sup>4</sup> where only occasional potassium determinations are carried out (α) five Gm. of cobaltous nitrate are dissolved in redistilled water, made up to 72.5 ml. with redistilled water and to this 2.5 ml. of glacial acetic acid is added. (β) 23 Gm. of sodium nitrite is dissolved in a small quantity of redistilled water and the volume made up to 75 ml.

Before use equal parts of solutions α and β are mixed with ice-cooling. For the rest of the procedure see above.

The air should be sucked through the mixture kept in a narrow tube. Both solutions can be kept on ice for a long time.

(2) N/100 solution of ceric sulfate commercial ceric sulfate has a variable content of crystal water and cerous salts. Therefore it is difficult to give exact directions for the preparation of a standard solution. The best method for obtaining a N/100 solution is as follows 10 Gm. of pulverized ceric sulfate is suspended in a small amount of water, 30 ml. of concentrated sulfuric acid is added (it

may have to be warmed) and it is diluted to 750 ml. with water. A crystal of KI and a few drops of starch solution are added to 2 ml. of this solution and the titer of the solution is determined with N/100 thiosulfate. The stock solution is diluted with the amount of water calculated from the titration to give N/100 ceric sulfate solution. It will keep indefinitely.

### Example

After the addition of 750 ml. of water 2 ml. of the ceric sulfate solution require 2.85 ml. of N/100 thiosulfate. According to the formula  $\frac{2}{2.85} = \frac{X}{100}$ , 702 ml. of the solution must be made up to 1000 ml.

Commercial ceric sulfate contains often acetic acid (acetic acid vapors may be noticed). In order to get rid of this impurity the powdered ceric sulfate is spread in a thin layer on the bottom of a wide Erlenmeyer flask. The flask is stoppered with a 2-hole stopper. Air, washed with sulfuric acid is sucked through the material until no more acetic acid odor can be detected. This procedure may be accelerated by immersing the flask in a boiling water bath.

(3) freshly prepared approximately 1 per cent KI solution

(4) 0.25 per cent starch solution

(5) N/100 sodium thiosulfate

### Procedure

One ml. of hemoglobin free serum is pipetted into a carefully cleaned pyrex centrifuge tube\* and 2 ml. of cobalt reagent (1) is added drop by drop with shaking and ice cooling. It is carefully overlaid with 1 ml. of redistilled water without mixing to prevent formation of a surface film of potassium-sodium cobalt-nitrate. The sample is left in ice water for 45-60 minutes to complete the precipitation, whereupon it is centrifuged at high speed for fifteen

The tubes are brushed with soap and water then they are placed in cleaning solution for twenty four hours rinsed carefully with water later with redist. water and dried at 40°C. Drying at higher temperature is to be avoided because the glass may get small grooves where the reagent can adhere during the determination. Quick drying can be achieved by centrifuging the cleaned tubes on absorbent cellulose in upside down position.



minutes. The supernatant is removed with the aid of the apparatus described for the Na-determination (fig 24). Then 0.5 ml. of ice-cold water is added to the precipitate and the water is carefully mixed with the remaining supernatant (avoid stirring up of the precipitate). After another portion of 5 ml. of water has been added carefully, the tubes are again centrifuged for 10–15 minutes. This procedure is repeated 2–3 times until the supernatant is colorless. After removal of the water as described above, 5 ml. of ceric reagent (2) is added and the tubes are heated in a boiling water bath for approximately two minutes to dissolve the precipitate. Thin glass rods are used for stirring. If a suitable metal rack for holding the tubes is available, all samples may be boiled at the same time. After cooling the contents of the centrifuge tubes are transferred quantitatively into Hagedorn-Jensen tubes. A few drops of KI solution and starch are added and the free iodine is titrated from a microburette with N/100 thiosulfate till colorless. When large centrifuge tubes are used (fig 24), the titration may be performed directly in the centrifuge tubes.

Exact potassium values can only be obtained when the serum is completely free of hemoglobin. *In order to avoid diffusion of potassium from the erythrocytes*—the potassium content of the red cells is approximately ten times that of serum—the serum must be separated immediately from the cells. After the blood has clotted it should be centrifuged immediately, only fresh sera may be used for the determination, as upon longer standing ammonia forms in the serum. Ammonia may also form an insoluble cobalt double salt, and thus interfere with the determination.

### Calculation

The potassium content of the sample in mg. is obtained by subtracting the amount of thiosulfate used for the sample from the thiosulfate value obtained in the blank run multiplied by 0.071. The blank value is equal to the thiosulfate used up by 5 ml. of N/100 ceric sulfate. The factor 0.071 has been determined empirically. Theoretically 1 ml. of N/100 thiosulfate corresponds to 1 ml. of

N/600 potassium solution (see reaction equation)  $\frac{39}{600 \times 1000} =$

0.065 mg potassium The factor 0.071 takes into account the solubility of the potassium cobaltic nitrite

### Example

Thiosulfate used up by the blank	4.80 ml
Thiosulfate used up by the sample	2.55 ml
Difference	2.25 ml

$2.25 \times 0.071 = 0.15975$  mg potassium in 1 ml of serum Multiplied by 100 = 15.975 mg per cent potassium in the analysed sample.

### Potassium Determination in Whole Blood

*Principle of the method* Potassium is determined in deproteinized whole blood as described above

### Reagents

(1) 7 per cent trichloroacetic acid

(2) Sodium nitrite solution 35 Gm of potassium free sodium nitrite are dissolved in 50 ml of redistilled water

All other reagents see determination of K in serum

### Procedure

The required number of narrow centrifuge tubes is filled with 2.8 ml of redistilled water from a pipet (see appendix, p 388). With an exactly calibrated pipet 0.2 ml of blood is taken from the finger tip or the ear lobe and delivered into the centrifuge tube. The pipet is rinsed several times by sucking up and blowing out the water in the tube. After the addition of 1 ml of trichloroacetic acid (1) the tube is centrifuged and 2 ml of the supernatant is carefully transferred to a dry centrifuge tube. If small protein particles should be floating in the supernatant filtering can be avoided by folding a small piece of quantitative filter paper around the tip of the pipet and sucking up the liquid through the pipet. Each sample receives 1 ml of sodium-nitrite solution (2) to neutralize the trichloroacetic acid. The tubes are well mixed and left at room temperature for several minutes. Then 2 ml of the cobalt reagent is added slowly. The remainder of the procedure is the same as described for serum and plasma.

*Calculation*

Thiosulfate used up for the blank determination minus thiosulfate required for the sample multiplied by 0.071 equals mg potassium in the sample

*Example*

thiosulfate used up by the blank	4.80 ml
thiosulfate used up by the sample (correspond to 0.1 ml of blood)	2.35 ml
difference	<hr/> 2.45 ml

The difference of 2.45  $\times$  0.071 results in a potassium content of 0.17395 mg in 0.1 ml of blood, multiplied by 1000 = 173.95 mg in 100 ml.

The normal serum potassium content is 16–18 mg per cent. Care must be taken in the potassium determination to separate immediately the serum from the cells in order to avoid diffusion of K from the red cells into the serum. Results obtained with hemolyzed blood are useless. The ratio of potassium content of serum to potassium content of whole blood is 1:10.

An increase in serum K is found in all processes which are accompanied by red cell destruction. In all these cases the potassium content of whole blood is decreased. An increased potassium level in whole blood with unchanged serum potassium is encountered in hyparglobulia. The serum potassium is increased in some cases of diabetes mellitus, stomach and duodenal ulcers, chronic arthritis, exudative tuberculosis, pleuritis, cirrhosis of the liver, hyperparathyroidism. A physiologic rise in serum K occurs in the premenstrual stage. During the menstrual period the potassium content falls and it returns again to normal in the postmenstrual period. Low serum potassium levels are encountered at times under pathologic conditions in decompensated heart failure, in obstructive icterus, and in cases of malignant tumors.

The potassium content of spinal fluid is practically identical with that of serum.

*AMMONIA DETERMINATION*

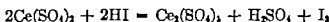
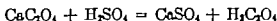
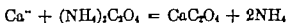
See determination of nonprotein nitrogen and its fractions, p. 170

## DETERMINATION OF CALCIUM IN SERUM

*Semimicro-method*<sup>5</sup>

*Principle of the method* Calcium is precipitated as calcium oxalate in serum or plasma. The oxalic acid bound to calcium is oxidized by the addition of a standard ceric sulfate solution to the dissolved precipitate and the excess ceric sulfate is determined iodometrically.

Reaction equation

*Reagents*

(1) Ammonium oxalate. An excess of finely pulverized ammonium oxalate is dissolved in distilled water. A saturated solution is prepared by shaking in the cold or by placing the mixture into an incubator for a short time.

(2) 4 N sulfuric acid. 11.6 ml of  $\text{H}_2\text{SO}_4$  concentrate is poured into water and made up with water to 100 ml.

(3) Ammonia solution. 2 ml of concentrated ammonia is diluted to 100 ml with distilled water or better (1) 0.5 ml. of triethanol amine is dissolved in 1 liter of water.

(4) N/200 ceric sulfate solution. commercial ceric sulfate has a variable content of crystal water and cerous salts. Therefore it is difficult to give exact directions for the preparation of a standard solution. The best method for obtaining a N/200 solution is as follows. 5 Gm. of pulverized ceric sulfate is suspended in a small amount of water. 30 ml. of concentrated sulfuric acid is added (it may have to be warmed up here), and it is diluted to 750 ml. with water. A crystal of KI and a few drops of starch solution are added to 2 ml. of this solution and the titer is determined with N/200 thiosulfate. The stock solution is diluted with the amount of water calculated from the titration to give N/200 ceric sulfate solution. It will keep indefinitely.

*Example*

After the addition of 750 ml of water, 2 ml of ceric sulfate solution require 2.85 ml of N/200 thiosulfate. According to the formula

$$\frac{2}{2.85} = \frac{x}{100} \quad 702 \text{ ml of solution must be made up to } 1000 \text{ ml}$$

(5) N/200 sodium thiosulfate solution

(6) 1 per cent KI solution

(7) 0.25 per cent starch solution

*Procedure*

A clean centrifuge tube (diameter 18–20 mm, height 85 mm) is filled with 1 ml of serum (or 0.5 ml.) and 1 ml of ammonium oxalate. It is well mixed and allowed to stand for twenty-four hours or heated for five minutes in a water bath of 56° C. After that time 1 ml of water is added by letting it run down along the walls of the tube to dissolve any ammonium oxalate crystals, and it is centrifuged for fifteen minutes at high speed. The supernatant is removed with a suction pump, using a capillary whose tip is bent to form a hook (see determination of sodium, fig. 24). The precipitate is stirred up with a thin glass rod or the centrifuge tube is tapped gently with tilting against soft wood or paper until the precipitate is well distributed. The glass rod and the walls of the tube are washed with 4 ml. of solution (3). The tube is again centrifuged for 8–10 minutes and the supernatant is removed as before. The washing with ammonia or triethanolamine is repeated twice more. Finally the precipitate is washed with redistilled water. After the last wash water has been removed, the oxalate precipitate is stirred up with the glass rod and dissolved in 0.5 ml. of sulfuric acid (2) in a boiling water bath (four minutes). While the solution is still warm 2 ml of N/200 ceric sulfate solution are added. After cooling to room temperature several drops of KI solution and starch are added and the solution is titrated to colorless with N/200 thiosulfate (b).

*Calculation*

Thiosulfate used for 2 ml of ceric sulfate minus thiosulfate used for the titration of the sample multiplied by 10 represents the calcium content of the blood in mg per cent. The factor is arrived at as

follows it can be seen from the equation that one atom of Ca corresponds to one molecule of oxalic acid i.e., to 2 atoms of iodine

$$\text{Consequently 1 ml. of a N/200 calcium solution equals } \frac{40}{2 \times 200 \times 100} \text{ Gm.} = 0.1 \text{ mg. Ca.}$$

Since 1 ml. of serum has been used for the analysis 1 ml. of N/200 thiosulfate corresponds to 10 mg. per cent of Ca.

### Example

Thiosulfate used up by the blank	1.99 ml
thiosulfate used up by the sample	0.98 ml
difference	1.01 ml

corresponds to 10.1 mg. per cent of calcium.

### SEMI-MICRO-METHOD FOR THE DETERMINATION OF CALCIUM IN LIPEMIC SERUM<sup>7</sup>

The method described above is not successful when lipemic serum is used. In order to obtain correct results for lipemic sera the proteins must be precipitated and the calcium determined in the deproteinized fat free filtrate.

### Reagents

- (1) 20 per cent trichloroacetic acid
  - (2) 20 per cent NaOH prepared from purest grade NaOH
  - (3) methylred-methyleneblue indicator 80 ml. of a saturated alcoholic solution of methylred is mixed with 20 ml. of a 1 per cent alcoholic methyleneblue solution
  - (4) 5 per cent acetic acid
- Other reagents as described on page 113

### Procedure

Four milliliters of serum are diluted with 12 ml. of redistilled water and the proteins are precipitated by the addition of 4 ml. of trichloroacetic acid (1). After a few minutes it is filtered through a quantitative ash free filter. Five ml. of the filtrate (corresponding to 1 ml. of serum) is transferred to a centrifuge tube as described above and 1-2 drops of indicator are added. Now NaOH is added in drops

until a faint green color appears. In order to prevent any precipitation of magnesium when ammonium oxalate is added, 1-2 drops of acetic acid are added until the green color changes back to red. One ml of ammonium oxalate is now added to the sample which is treated as described above

#### CALCIUM DETERMINATION IN 0.2 ML. OF SERUM

Calcium is precipitated with ammonium oxalate and after the excess ammonium oxalate has been removed by repeated washing the precipitate is dissolved in sulfuric acid. The liberated oxalic acid is oxidized by the addition of a known amount of a standard N/1000 ceric sulfate solution and the excess ceric sulfate is titrated with N/1000 thiosulfate

Reaction equation (see p 113)

#### *Reagents*

(1) ammonium oxalate solution, cold saturated before each analysis the required amount of ammonium oxalate solution is freed from crystals by centrifugation and the supernatant is removed with a pipet

(2) approximately 4 N  $H_2SO_4$ , prepared by diluting 11.6 ml of concentrated  $H_2SO_4$  to 100 ml. with redistilled water

(3) ammonia solution 2 ml of concentrated ammonia is diluted to 100 ml. with redistilled water

(4) N/1000 ceric sulfate solution prepared by diluting 100 ml of N/200 ceric sulfate to 500 ml with redistilled water (N/200 ceric sulfate solution see p 113)

(5) 1 per cent KI solution

(6) 0.25 per cent starch solution

(7) N/1000 sodium thiosulfate solution (preparation see chapter VII p 250)

#### *Procedure*

With an exactly calibrated pipet 0.2 ml of serum (plasma) is measured out and blown into a centrifuge tube (see semi micro method) which has been filled with 0.5 ml of ammonium oxalate solution (1). To complete the precipitation the sample is left over night at room temperature or heated for five minutes in a water bath of  $56^\circ C$ . Then the walls of the tube are washed down with approxi-

mately 1 ml of redistilled water to remove any ammonium oxalate crystals and it is centrifuged for fifteen minutes at 3000 r.p.m. The supernatant is carefully removed by suction (water pump) with the aid of a capillary pipet bearing a hook-shaped tip (fig 24). Care must be taken not to stir up the precipitate. Three ml of ammonia solution are added the tubes again centrifuged and the supernatant removed as described above. This procedure is repeated twice more. The precipitate must not be stirred up during the process of washing. Now the precipitate is dissolved in 0.5 ml of sulfuric acid while stirring with a thin glass rod, to accelerate the dissolving the sample is heated in a boiling water bath. After cooling to room temperature each sample receives 2 ml. of ceric sulfate and is allowed to stand for at least three minutes. With a few drops of KI solution and starch the sample is titrated to colorless with N/1000 thiosulfate.

### *Calculation*

Thiosulfate required for 2 ml. of ceric sulfate solution minus thiosulfate used up by the sample multiplied by 10 results in the calcium content of the serum in mg per cent (calculation in detail see under *calculation*, p. 114)

Since the red blood cells are free from calcium there is no need to determine the calcium in whole blood.

The normal calcium content of serum is 8-11 mg per cent, that of plasma is usually 0.5-1 mg per cent lower. The calcium in serum exists in two physiologically different forms the Ca which can be dialyzed and the Ca which cannot be dialyzed and which is probably combined with the serum proteins. The dialyzable form—probably the physiologically active fraction—amounts to approximately 4.5-5.5 per cent of the total Ca content of the serum (4.5-5.5 mg per cent). The dialyzable form is practically identical with the calcium content of spinal fluid.

High Ca levels are found in

- (1) hyperparathyroidism. After injection of parathyroid hormone the calcium level will rise.
- (2) osteitis fibrosa (Recklinghausen's disease) (12-19 mg. per cent)
- (3) during hypervitaminosis (vitamin D) after ingestion of the vitamin preparation A.T. 10
- (4) some cases of nephritis with uremia



- (5) multiple myeloma
- (6) polycythemia vera (unknown reason) 14-18 mg per cent
- (7) extensive emphysema

Low calcium levels occur in

- (1) hypoparathyroidism
  - (a) infantile tetany and spasmodophilia often connected with rickets
  - (b) tetany together with osteomalacia
  - (c) idiopathic juvenile tetany
  - (d) tetany after parathyroidectomy, sometimes after thyroidectomy
- (2) vitamin D deficiency in many cases normal Ca levels and low serum phosphate levels may be found
- (3) sprue, kala azar
- (4) kidney diseases

(a) nephroses (5.7-9.1 mg per cent) characterized by low non-dialyzable Ca with normal values for dialyzable Ca

(b) nephritis in cases which do not tend to form edema a low Ca level is occasionally encountered. In advanced cases with uremic symptoms the Ca level can drop to 4-6, mg per cent and at the same time the inorganic phosphorus may rise to 12-20 mg per cent

- (5) hypoproteinemia

The Ca content of spinal fluid corresponds to the value obtained in serum ultrafiltrates.

## MAGNESIUM DETERMINATION

### *Colorimetric Determination\**

*Principle of the method* The calcium is precipitated with ammonium oxalate and then the magnesium as magnesium-ammonium phosphate. The phosphorus content of the precipitate is determined and from this value the magnesium content of the original sample is calculated.

### *Reagents*

- (1) cold saturated ammonium oxalate solution
- (2) 2 per cent ammonium phosphate solution
- (3) concentrated ammonia

Mainly according to W. Dennis Journ Biol Chem 55 411 1922.

(4) dilute (2 per cent) ammonia solution 10 ml of concentrated ammonia is diluted with water to 100 ml

(5) 10 N sulfuric acid, prepared by adding 279 ml of concentrated  $H_2SO_4$  to about 500 ml of redistilled water and making up to 1000 ml after cooling

(6) sodium molybdate reagent 7.5 Gm of sodium molybdate (analytical reagent) are dissolved in a 100 ml volumetric flask in redistilled water and made up to the mark with water Should this solution be cloudy it must be filtered through an ash free filter

(7) stannous chloride reagent

(a) stock solution in a 25 ml volumetric flask 10 Gm of  $SnCl_2$  are dissolved in a few ml of concentrated HCl and the volume is made up to the mark with concentrated HCl A few pieces of metal tin (phosphorus-free)<sup>9,10</sup> are added to this solution to prevent autoxidation of  $SnCl_2$  according to the formula  $SnCl_2 + Sn = 2SnCl_2$  This solution when stored under refrigeration will keep indefinitely (see determination of phosphorus, p 94)

(b) working solution before use the stock solution is diluted the degree of dilution is found by determining the concentration of the stock solution. 0.1 ml of  $SnCl_2$  is placed into a 50 ml volumetric flask and filled up to the mark with distilled water Five ml of N/200  $KIO_3$  are placed into test tubes (diameter 2.5 cm), KI is added all is well mixed and 1 ml of dilute  $SnCl_2$  solution is added. After some seconds the liberated iodine is titrated with N/200 thio-sulfate till yellow and after adding some starch the titration is continued until the blue color disappears. In the same way 2 blank values are titrated without  $SnCl_2$ . The difference between blank and full value multiplied by 0.5625 =  $\frac{225000 \text{ (mol. } SnCl_2\text{)}}{2 \times 200 \times 1000}$  gives mg  $SnCl_2$  in 1 ml of the dilute solution According to Kuttner and Laechtenstein the  $SnCl_2$  solution should contain 2 mg  $SnCl_2$  per ml From the titration results the dilution factor is calculated and the solution diluted accordingly

*Example*

blank value needs

full value needs

50 ml thio-sulfate

28 ml thio-sulfate

difference

---

22 ml

$2.2 \times 0.5625 = 1.2375$

1 ml of this solution thus contains 1.23 mg per cent  $\text{SnCl}_2$ . From the proportion  $\frac{0.1}{x} = \frac{1.23}{2}$  the amount of  $\text{SnCl}_2$  solution is found

which must be diluted to 50 ml. In this case ( $x = \frac{0.2}{1.23} = 0.16$ )

0.16 ml must be diluted to 50 ml. As the concentrated solution is nearly constant, it should be controlled once in a time, respectively every time before use, if the estimation is carried out only occasionally.

If only occasional Mg or P determinations are to be performed it is advisable to prepare the working solution which will keep only for one day, shortly before use as follows: on an ordinary balance 0.2 Gm  $\text{SnCl}_2$  is weighed, dissolved in a 200 ml volumetric flask in 1 ml of concentrated HCl and made up to the mark with redistilled water.

#### (8) phosphoric acid standard solution

(a) stock solution 1.5256 Gm  $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$  (secondary sodium phosphate according to Soerensen) or 1.004 Gm.  $\text{KH}_2\text{PO}_4$  (weighed on an analytical balance) is dissolved in a 500 ml volumetric flask in some 10 N  $\text{H}_2\text{SO}_4$  (5) and filled up to the mark with the same acid. This solution will keep for a long period of time.

#### (b) working standard

(a) for the Dubosque colorimeter Forty five ml of 10 N  $\text{H}_2\text{SO}_4$  is added to 5 ml of stock solution in a 100 ml volumetric flask and the volume is made up to the mark with water. Two ml of this dilute standard solution correspond to 0.5318 mg of phosphorus or 0.04172 mg of magnesium.

(b) for the Hellige colorimeter Forty ml. of  $\text{H}_2\text{SO}_4$  (5) are added to 10 ml of the stock solution in a 100 ml. volumetric flask and made up to the mark with water. Two ml of this solution correspond to 0.10636 mg of phosphorus or 0.08344 mg of magnesium.

#### (9) N/1 NaOH

#### Procedure

Two ml of serum (plasma), 3 ml. of redistilled water and 1 ml of ammonium oxalate solution are placed in a centrifuge tube and left over night at room temperature (to complete the Ca precipitation). After centrifuging 5 ml of the clear supernatant are trans-

ferred to a centrifuge tube marked at a point indicating 8 ml. The precipitate may be used for Ca determination (see p. 113). To the supernatant 1 ml. of ammonium phosphate solution and 2 ml. of ammonia are added, well mixed with a thin glass rod, and the tube is placed into a water bath of 80° C for five minutes. Then the tube is lightly corked and allowed to stand over night. The next day it is centrifuged at high speed and the supernatant is removed as described for Na-determination (p. 100). Now 6 ml. of 2 per cent ammonia are added to the precipitate of magnesium ammonium phosphate the tube is again centrifuged and the supernatant carefully removed. This procedure is repeated twice more. After the last separation 1 ml. of N/1 NaOH is added to the precipitate and the tube is heated in a boiling water bath until red litmus paper does not turn blue when exposed to the vapors. One ml. of 10 N H<sub>2</sub>SO<sub>4</sub> is slowly added to the hot sample to dissolve the precipitate. After cooling it is made up to the mark with redistilled water, at the same time 2 ml. of dilute standard solution in a similar tube are diluted with redistilled water to 8 ml. To the unknown sample and to the standard 1 ml. of sodium molybdate solution and 1 ml. of dilute SnCl<sub>2</sub> solution is now added. After approximately two minutes the maximum color intensity is established and the color will remain stable over a considerable period of time.

*Calculation* (a) for the Dubosque colorimeter

$$\frac{\text{reading of the standard} \times 0.01172}{\text{reading of unknown sample}} = \text{mg. of Mg in } \frac{5 \times 2}{6} \text{ ml. of serum.}$$

$$\frac{\text{reading of the standard} \times 0.01172 \times 6 \times 100}{\text{reading of unknown sample} \times 10} = \frac{\text{reading of the standard} \times 2.5}{\text{reading of unknown sample}} = \text{mg. per cent magnesium}$$

*Example*

reading of standard	20.0 mm.
reading of unknown	17.5 mm.
difference	2.5 mm.

the magnesium content of the analyzed sample is  $\frac{20 \times 2.5}{17.5} = 2.8$  mg per cent.

*Calculation* ( $\beta$ ) for the Hellige colorimeter

$$\frac{100 - y}{100} \times 0.08344 = \text{mg. magnesium in } \frac{2 \times 5}{6} \text{ ml. serum.}$$

$y$  = colorimeter reading

$$\frac{(100 - y) \times 0.08344 \times 6}{100} = \text{mg. magnesium in 10 ml. of serum}$$

$$\frac{(100 - y) \times 0.50064}{100} = \text{mg. magnesium in 10 ml. of serum}$$

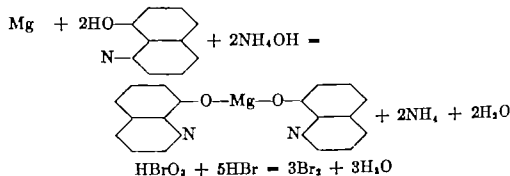
$$\frac{(100 - y) \times 5}{100} = \text{mg. per cent Mg.}$$

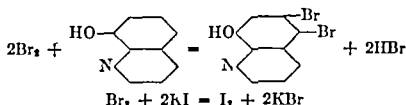
*Example*

$$\begin{aligned} \text{colorimeter reading 44, } & \frac{(100 - 44) \times 5}{100} \\ & = \frac{56 \times 5}{100} = 2.8 \text{ mg per cent Mg} \end{aligned}$$

#### *Titrimetric Magnesium Determination<sup>15-16</sup>*

*Principle of the method* Magnesium is precipitated with oxyquimoline in the form of an insoluble complex salt  $\text{Mg}(\text{C}_8\text{H}_8\text{ON})_2 \times 2\text{H}_2\text{O}$ . At a certain pH and temperature the calcium remains in solution. The precipitate of Mg-o-oxyquimoline is dissolved in HCl and the oxyquimoline content is determined by bromination



*Reagents*

- (1) 20 per cent trichloroacetic acid
- (2) concentrated ammonia
- (3) ammonium chloride p.a.
- (4) 8-ortho-oxyquinoline, 4 per cent solution in 96 per cent ethanol freshly prepared for each analysis
- (5) 5 per cent ammonium acetate solution (with ammonia) 5 Gm of ammonium acetate is dissolved in 100 ml of distilled water and enough ammonia is added to obtain an alkaline reaction to phenol phthalein
- (6) dilute HCl
- (7) 50 per cent potassium bromide solution
- (8)  $\sim$ /100 potassium bromate solution 278 mg of  $\text{KBrO}_3$  is dissolved in 1 liter of water
- (9)  $\sim$ /200 sodium thiosulfate solution
- (10) KI crystals
- (11) 0.25 per cent starch solution

*Procedure*

In a centrifuge tube 3 ml of serum are diluted with 3 ml of distilled water, 3 ml of trichloroacetic acid is added and the tube is centrifuged at high speed. Three ml of the clear supernatant (corresponding to 1 ml of serum) are removed with a pipet and transferred to a clean, dry test tube. To this 0.1 Gm of ammonium chloride is added and the tube is placed into a water bath of  $80^\circ\text{C}$  for five minutes. Two tenths (0.2) ml of a freshly prepared 4 per cent oxyquinoline solution and approximately 0.1 ml of ammonia are then added to the warm solution (until the color changes from light yellow to dark yellow). To speed up the precipitation of the magnesium quinoline complex the solution is well mixed for one minute with a thin pyrex glass rod or a platinum wire. The sample is again placed into a water bath of  $80^\circ\text{C}$  and it is slowly brought to boil.

The tube is left in the boiling water bath for 1 minute. Now the sample is filtered through a Pregl filter (fig 25a) while still hot.

To avoid the formation of a precipitate along the walls of the filter tubing the apparatus is rinsed with hot water immediately before use. The filtration is performed as follows. The test tube containing the precipitate is connected to the filtering apparatus and the precipitate is removed with the siphon. The tube is washed 2-3 times with hot ammonium acetate solution and the wash water is filtered in the same manner. Then the filter is removed from the suction flask and placed upon a clean wide-mouthed tube (Hagedorn Jensen flask) or any suitable tube and secured by way of a 2-hole rubber stopper (fig 25b). The precipitate is now dissolved in dilute HCl. Any magnesium precipitate adhering to the walls of the test tube

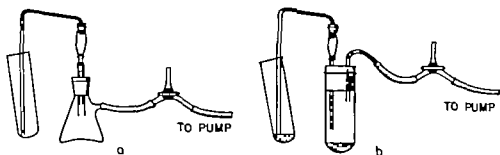


FIG 25 EQUIPMENT USED FOR SUCTION

is washed twice with 2 ml each of hot HCl and this acid is also sucked through the filter. The filter is removed from the tube (or flask) and the stem is rinsed with water. The filtrate (magnesium salt solution) is allowed to cool, an accurately measured amount of potassium bromate solution (2-3 ml) and 1 ml of potassium bromide solution are added, the vessel covered with a watch glass and left at room temperature for several minutes. After the watch glass has been rinsed off with water, a few crystals of KI and a few drops of starch solution are added and the liberated iodine is titrated with thiosulfate till colorless.

(Preparation of the filter tubes. The empty filter tubes are filled with dry clean asbestos, the asbestos is pressed together with a sharp-edged bent glass rod care being taken that the asbestos paste fills up the entire space of the filter especially along the glass wall. Now the filter is placed upon a suction flask and finely suspended

asbestos is sucked through with the water pump. The filter is now washed successively with chromic acid, sulfuric acid, water, dilute hydrochloric acid, ammonia. Before each determination hot water should be filtered through as mentioned above.)

### Calculation

Thiosulfate required for the blank (2-3 ml of bromate solution + dil. HCl + 1 ml of KBr) minus thiosulfate required for the sample multiplied by 0.0152 gives the magnesium content of the sample in mg. The factor 0.0152 is obtained from the formula according to which 8 bromine atoms correspond to 1 magnesium atom. If 1 ml of serum is used 1 ml of a N/200 sodium thiosulfate solution corresponds to  $\frac{24.32}{8 \times 200 \times 1000} = 1.52$  mg per cent magnesium.

### Example

blank value (thiosulfate required by 3 ml of $\text{KBrO}_3 + \text{KBr} + \text{HCl}$ )	5.60 ml
full value (thiosulfate required by the sample)	3.90 ml
difference	1.90 ml

$1.90 \times 1.52 =$  magnesium content of 2.888 mg per cent

The normal magnesium content of serum is 2.0-2.8 mg per cent, that of whole blood is 3.0-4.0 mg per cent. The magnesium level rises during sleep or after heavy physical labor. There is no rise in magnesium level in cases of poisoning by sedatives. A high magnesium level can be encountered in myasthenia and during periodic paralysis of the extremities which may be connected with an occasionally occurring thyrmus persistence and in uremia. A small rise may be found in hypertension, hypertrophic arthritis and after the injection of parathyroid hormone. This rise reaches its maximum ahead of the calcium peak and subsides again after one hour.

The magnesium content of spinal fluid is somewhat higher than that of serum.

## IRON DETERMINATION<sup>13</sup>

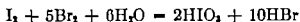
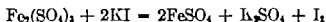
### Iron Determination in Whole Blood

*Principle of the method* By digestion of the blood with hydrogen peroxide the iron is converted into the ferric form. The digestion



residue is transferred into the Parnas-Wagner apparatus which has been modified for this purpose (see fig 47). After a potassium iodide solution has been added, the iodine liberated by the reduction of ferric iron to ferrous iron is steam distilled into sodium hydroxide plus sodium sulfite. The sodium iodide is oxidized to iodate and titrated with thiosulfate after the addition of KI.

Reaction equation



### Reagents

(1) 2 Gm. of ammonium sulfate and 0.05 Gm. of titanium hydroxide are suspended in 50 ml. of water and dissolved by the addition of 33 ml. of iron free concentrated sulfuric acid. When cool the solution is made up to 100 ml. with water.

(2)  $\text{H}_2\text{O}_2$ , 30 per cent (superoxol)

(3) potassium-cadmium iodide. 20 Gm. of cadmium sulfate is dissolved in water and made up to 100 ml. This solution will keep indefinitely. Before use 1 Gm. of KI (purest grade, iodate-free) is dissolved in 10 ml. of the cadmium sulfate solution.

(4) (a) N/1 NaOH

(b) 2 per cent sodium sulfite solution (will keep only for a short while). Before use 20 parts of solution (a) are mixed with 15 parts of solution (b).

(5) sulfuric acid for neutralization. In a 1000 ml. volumetric flask 11 ml. of concentrated  $\text{H}_2\text{SO}_4$  is poured into some water and made up to the mark after cooling.

(6) indicator. 15 mg. of methylred is dissolved in 10 ml. of N/1 NaOH and made up with water to 1000 ml.

(7) sodium acetate solution, 15 per cent

(8) bromine in glacial acetic acid. 6 ml. of bromine for 100 ml. of glacial acetic acid.

(9) concentrated formic acid

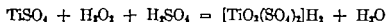
(10) potassium iodide

(11) N/200 sodium thiosulfate solution

(12) 0.25 per cent starch solution

*Procedure*

With a capillary pipet 0.1–0.2 ml of blood is drawn from the finger tip (or oxalated blood is used). The outside of the pipet is wiped clean and the contents are delivered into a microkjeldahl flask, containing several glass beads to prevent bumping (a small piece of glass wool may be used instead of the beads, the glass wool must be washed first with cleaning solution and then with distilled water and it must be thoroughly dried). The pipet is rinsed with a few drops of water. After the addition of a few drops of the digestion acid (1) and 0.1 ml of  $\text{H}_2\text{O}_2$  the flask is heated slowly at first and later on stronger until the white sulfuric acid fumes disappear. The digestion is interrupted at this point the mixture is cooled. 1 ml of superoxol is added and the flask is again heated until the orange-red color (sign of  $\text{H}_2\text{O}_2$ ) has disappeared. Titanium sulfate and  $\text{H}_2\text{O}_2$  will form a red pertitanic acid [peroxodisulfato-titanic acid] according to the formula



After cooling the residue is diluted with redistilled water. If a red color appears again due to traces of  $\text{H}_2\text{O}_2$  left on the neck of the flask the digestion must be repeated as  $\text{H}_2\text{O}_2$  interferes with the reaction (oxidation of potassium iodide). Now the sample is transferred quantitatively into the apparatus with the aid of the funnel (b) (see fig 47 p 274). The stopcock is closed and steam is blown through the apparatus from the flask (m) until all air has been expelled. This can be recognized by the fact that the tube gets hot at (k) and no more air bubbles escape through the receiver. With the stopcock in suitable position 2 ml of potassium-cadmium iodide (3) are added drop by drop through the funnel. Two ml. of the alkaline sodium sulfite solution (4) are placed into the receiver flask. The distillation is carried out for 2 minutes with the tube (1) reaching under the surface of the liquid in the receiver flask. After that time the receiver is lowered and the distillation is continued for two more minutes. At the end of the distillation 5 drops of the indicator (6) are added to each receiver flask and dilute sulfuric acid (5) is added drop by drop till the reaction is faintly acid. To each flask 3 ml of sodium acetate and bromine-glacial acetic acid is added from a dropping flask until the brown color (bromine) persists. After

1-2 minutes formic acid is added in drops until the bromine color disappears. The bromine vapors (if present) are removed by suction with a water pump and 2 ml of sulfuric acid (5) is added. After the addition of a few crystals of KI and starch solution as indicator the liberated iodine is titrated with N/200 thiosulfate to colorless.

After each determination the apparatus must be washed with redistilled water, the water being sucked through the inner tube of the condenser with a water pump.

If organs are to be digested 1 ml of concentrated nitric acid is added to the digestion acid and after the water has evaporated the sample is treated as described above.

The digestion may also be performed as follows, omitting the  $H_2O_2$  and titanium hydroxide which is difficult to obtain in a form readily soluble in acids.

Instead of reagent (1) reagent A is used. 33 ml of iron free  $H_2SO_4$  concentrate is added to water in a 100 ml volumetric flask and after cooling made up to the mark with water.

Instead of reagent (2) the following reagents are used:

reagent B)  $HNO_3$  concentrate p.a. and

reagent C) 10 per cent urea solution

otherwise as described above. The digestion is modified as follows. With a capillary pipet 0.1-0.2 ml. of blood are drawn from the finger tip (or oxalated blood is used), the outside of the pipet is wiped clean and the blood is delivered into a micro-Kjeldahl flask, containing several glass beads. The pipet is rinsed with redistilled water. After the addition of 2 ml of the digestion mixture (A) and 1 ml. of  $HNO_3$  (B) the flask is heated gradually, until the nitric acid and sulfuric acid fumes have disappeared. If the residue is still colored after cooling, 1 ml of  $HNO_3$  is added and the digestion repeated. After cooling 2 ml of urea (C) are added to destroy any possibly present nitrous acid. The contents of the flask are then carefully transferred into the funnel of the Parnas-Wagner apparatus (fig. 47, funnel b) and the procedure is continued as described above.

### *Calculation*

Thiosulfate value for the sample minus value obtained for the blank multiplied by 0.0466 gives mg iron in the unknown sample. (Together with the analysis 1 or 2 blank determinations are carried

out. If the analysis is done carefully and if only the purest grade reagents have been used, no iodine is liberated in the blank runs and the blank value is 0. The factor 0.0466 is calculated from the reaction equation, which shows that 1 atom of iron corresponds to 6 atoms of iodine. Consequently 1 ml. of N/200 thiosulfate solution is equal to  $\frac{55.84}{6 \times 200 \times 1000} = 0.0466$  mg Fe. It is multiplied by 1000 to obtain the result in mg per cent. For 0.1 ml of blood being analyzed, one ml of N/200 sodium thiosulfate corresponds to 46.6 mg per cent; for 0.2 ml of blood the corresponding concentration is 23.3 mg per cent.

### Example

0.2 ml of blood require 2.15 ml of N/200 thiosulfate, blank value = 0. Iron content of the blood =  $2.15 \times 23.3 = 50.09$  mg per cent.

### Iron Determination in Serum

*Principle of the method.* The total non-hemin iron of serum is converted into the ionized form and can be found in the deproteinized filtrate of the trichloroacetic acid precipitation. After the reduction with hydroquinone the iron is determined colorimetrically at pH 4.0-4.2 as ferrous-phenantroline complex salt.

*Method (a) according to Heilmeyer and Ploetner<sup>14,17</sup>*

### Reagents

(1) 6 N HCl. HCl conc. p.a. contains traces of iron and can be purified by distillation over concentrated  $H_2SO_4$  and absorbing the HCl gas in distilled water. The distilling apparatus must be made entirely of glass.

(2) Twenty per cent trichloroacetic acid. The purest grade of  $CCl_3COOH$  contains traces of iron and can be purified by vacuum distillation.

(3) One per cent phenantroline or dipyridyl solution (p. 263). 1 Gm. + 100 ml of water, 1 drop of concentrated  $H_2SO_4$  added.

(4) Two per cent hydroquinone solution. 2 Gm. of pure hydroquinone in 100 ml of water plus 1 drop of concentrated  $H_2SO_4$ . This solution will keep for a few weeks when stored in a dark bottle in a refrigerator; it must be discarded when a brown color appears.

(5) One per cent nitrophenol 1 Gm. of p-nitrophenol in 100 ml. of 90 per cent alcohol kept in a dropping bottle

(6) Twenty per cent ammonia p.a.

(7) 0.5 N  $H_2SO_4$  prepared by diluting 14 ml. of concentrated  $H_2SO_4$  in 100 ml. of water

(8) acetate buffer solution pH 4.0-4.2 25 ml. of glacial acetic acid and 25 Gm. of sodium acetate are made up to 100 ml. with water. Test 5 ml. of buffer solution should not give a yellow color with 1 drop of p-nitrophenol

(9) Iron standard solution to contain 1  $\gamma$  of iron per ml. 0.7023 Gm. of ferrous ammonium sulfate (Mohr's salt) are dissolved in 100 ml. of 1 per cent  $H_2SO_4$  in a 1 liter volumetric flask and made up to the mark with water. One ml. of this solution is placed into a 100 ml. volumetric flask, acidified with a few drops of HCl conc. and made up to the mark with distilled water

Since the amount of iron to be determined is only 1-2  $\gamma$  the analysis must be performed very carefully and all contamination with iron has to be avoided. All glass ware used must be treated with HCl and rinsed with distilled water. The filter paper must be iron free. A hard filter (Whatman Nr. 30) which is used may contain traces of iron and should be washed several times with 10 per cent HCl and water. After suitable washing the same filter may be used over again.

Blank determination if the determination is performed according to the description using 2 ml. of water instead of serum the color of the blank should not exceed the color obtained by mixing 0.3  $\gamma$  of iron in 5 ml. of water with 1 drop of phenantroline and 1 drop of hydroquinone.

### *Procedure*

One ml. of HCl (1) is added to 2 ml. of serum or plasma (slight hemolysis will not interfere) in a small Erlenmeyer flask or test tube, well mixed and allowed to stand for ten minutes. After the addition of 2 ml. of trichloroacetic acid (2) the flask is left standing for another ten minutes whereupon the mixture is transferred to a centrifuge tube and centrifuged. The supernatant is filtered through a small iron free filter into a graduated 10 ml. glass tube. Between 3.2 and 3.5 ml. of filtrate are obtained. 0.05 ml. of phenantroline solution

(3) 0.1 ml. of hydroquinone solution (4) and 0.05 ml. of nitrophenol (5) are added to the tube well mixed and ammonia (6) is added slowly drop by drop. At first the pink color of the phenantroline compound appears, after a few more drops the definite deep yellow color of the indicator can be observed. At this point sulfuric acid (7) is added drop by drop until the yellow color disappears and the pink color is again visible. After the addition of 0.2 ml. of acetate buffer (8) the volume is made up with water to 5 ml. and the color is read after five minutes. The color will remain constant for several hours and the reading need not be done immediately. Two ml. of standard solution (9) in a centrifuge tube are mixed with 1 ml. of HCl (1) and 2 ml. of trichloroacetic acid (2), then centrifuged and filtered. The same amount of this filtrate as serum filtrate obtained is treated exactly as described above.

Standard and sample are read in the colorimeter in a microtube through a 30-50 mm. deep layer. The accuracy of the reading can be enhanced by inserting a green filter (absorption at about 500  $\mu$ ). If the blank values fall below the indicated figure amounts down to 40  $\gamma$  per cent may be determined with an accuracy of 5-10 per cent.

#### *Example*

reading of the standard in the colorimeter = 30 mm  
 reading of the unknown sample = 26.4 mm.

$$\frac{30 \times 100}{26.4} = 114\gamma\%$$

For better reading see electro-photocolorimeter, p. 398

*Method (b) according to Barkan and Walker<sup>13</sup>*

#### *Reagents*

- (1) HCl 1.2 per cent aqueous solution
- (2) trichloroacetic acid 10 per cent aqueous solution
- (3) sodium acetate saturated solution
- (4) 2 M acetate buffer pH 4.5 prepared by mixing 90 vol. of 2 M sodium acetate and 110 volumes of 2 M acetic acid
- (5) hydrazine sulfate 1 per cent solution in 2 M acetate buffer pH 4.5. This solution must be freshly prepared daily
- (6) o-phenanthroline monohydrate 0.1 per cent solution in water

The preparation may be warmed to accelerate solution. It should be discarded if any color appears.

(7) Iron standard see under *method a*) p. 129

*Procedure* 2 ml. of serum or plasma are measured into a short rimless test tube (not a conical centrifuge tube, on account of the later difficulty in removing the precipitate). One ml. of 1.2 per cent HCl is added, the tube stoppered and kept at 37° C for one hour. When cooled to room temperature, 1 ml. of 20 per cent trichloroacetic acid is added, mixed cautiously but thoroughly and left standing for one hour at room temperature. The tube is covered with tin foil and centrifuged (3000 r.p.m.) for fifteen minutes. To 2 ml. of clear supernatant fluid are added in succession 0.5 ml. of saturated sodium acetate solution, 0.5 ml. of buffered 1 per cent hydrazine sulfate solution and 0.5 ml. of 0.1 per cent o-phenantroline monohydrate solution. Two ml. of iron standard are treated in the same way. Proceeding and calculation see under *method a*).

Traces of hemoglobin in the serum or plasma will not introduce an error in this procedure. Hemoglobin is precipitated with trichloroacetic acid. The iron of pseudohemoglobin (about 4 per cent of the hemoglobin iron) is split off by the incubation and appears in the final result. This is however of significance only in grossly hemolyzed sera.

The iron content of whole blood of a normal person varies between 45 and 50 mg. per cent; it is dependent upon the hemoglobin content and it is elevated in polyglobulia and lowered in anemia.

The iron content of serum in a normal male is approximately 130  $\gamma$  per cent; in females approximately 100  $\gamma$  per cent. It varies considerably in normal persons (80–175  $\gamma$  per cent in males, 70–150  $\gamma$  per cent in females). There are only a few figures available for children, but it seems as if the values tend to be lower than those for adults. Values below 65  $\gamma$  per cent and above 190  $\gamma$  per cent for males and below 50  $\gamma$  per cent and above 175  $\gamma$  per cent for females must be considered pathologic. The serum level of iron is lowered in all anemias with iron deficiency, often in pernicious anemia during remission and in various infectious diseases. It is elevated in hemolytic processes, shortly after transfusions and after massive therapeutic iron dosage, often in pernicious anemia and in aplastic anemia.

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## Chapter V

# Determination of the Nonprotein-Nitrogen and its Fractions

As nonprotein nitrogen are classified all nitrogen compounds of blood (serum, plasma) which are not precipitated by protein precipitating agents. The most important of these compounds are urea ammonia, amino acids (free and combined), uric acid creatine and creatinine, indican, undetermined nitrogen

### DETERMINATION OF NONPROTEIN NITROGEN

#### *Titrimetric Semi Micromethod in Serum and Plasma with Distillation*

*Principle of the method* In the protein free filtrate the amido-bound nitrogen is converted into ammonia. The ammonia is liberated by strong alkali and is distilled into a known amount of hydrochloric acid, containing potassium iodate. The excess HCl is determined iodometrically.

#### *Reaction equation*



The excess HCl is determined iodometrically according to



#### *Reagents*

- (1) 20 per cent trichloroacetic acid
- (2) copper sulfate-sulfuric acid mixture 10 Gm of copper sulfate is dissolved in 500 ml of water and to this solution is added carefully and with cooling 500 ml of concentrated  $\text{H}_2\text{SO}_4$
- (3) 33 per cent KOH (ammonia free) Kjeldahl alkali
- (4) iodate-hydrochloric acid In a volumetric flask 200 ml of  $\text{N}/10$  HCl or  $\text{N}/10$   $\text{H}_2\text{SO}_4$  are mixed with 100 ml of a 4 per cent

potassium iodate solution and made up to 1000 ml with distilled water. The solution will keep for a long period of time.

(5) N/100 neutral sodium thiosulfate solution

(6) KI crystals

(7) 0.25 per cent starch solution

A modified Parnas-Wagner apparatus<sup>1,2</sup> (fig 26) is used for the determination of non protein nitrogen according to this method, it is also used for the determination of urea (p 153)

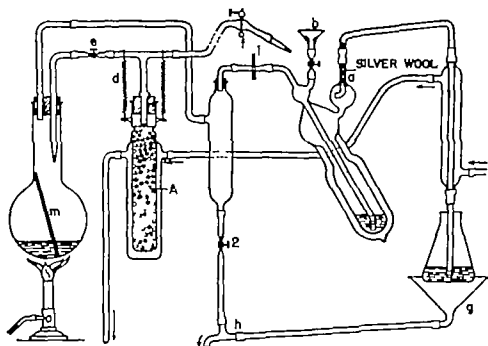


FIG 26 Modified Parnas-Wagner distilling apparatus for the determination of nonprotein nitrogen and urea

Traces of  $H_2S$  and other sulfur compounds originating from the rubber tubing can interfere seriously with the distillation of small amounts of ammonia such as occur in these determinations. To exclude this source of error the ascending part (a) of the condenser following the last rubber tubing connection is filled with silver wool. Any possible traces of  $H_2S$  are retained through  $Ag_2S$  formation. No metal condensers must be used in this method. It is most important that the connection between distilling flask and condenser consists of a well fitting ground glass joint. A glass vessel A is

inserted between steam generator and condenser which is filled with sodium permutoxide and which is closed at the bottom with a perforated glass plate or gooch crucible plate and closed at the top with a porcelain filter plate. This vessel is surrounded by a glass jacket C, bearing 2 side arms. The water coming from the condenser surrounds the permutoxide vessel and drains without making contact with the permutoxide. Both vessels are closed at the top with a one-hole rubber stopper through which leads a T tube. One arm of the T tube (b) is connected with the steam generator, the current of water being regulated with a clamp. In this manner a constant water level in the generator is maintained. The other arm of the T tube (f) is used to wash the digestion flask. The water used for washing or for refilling of the generator is forced through the permutoxide vessel (A) and is thus freed from ammonia. This apparatus permits the use of tapwater for the determination. A small amount of silver wool may be added to the permutoxide in order to avoid the growth of algae in the ammonia rich permutoxide or along the walls of the water jacket (oligodynamic effect of silver).

The apparatus must be steamed out before each analysis. To this purpose clamp 1 is opened, clamp 2 is closed. Between determinations it suffices to wash the apparatus with water, the flame is removed from the steam generator and the resulting negative pressure will suck the liquid from the distilling flask into the collector. A few ml. of water are introduced through the funnel T and again removed by suction. The washing is repeated 2-3 times. Now the flame is replaced under the steam generator and at the same time clamp 1 is closed and clamp 2 is opened. The sample is filled into the distilling flask through the funnel (b) and the receiver flask is placed below the condenser tube, the tube reaching below the surface of the liquid. clamp 1 is opened and at the same time clamp two is closed. A large funnel (g) serves to collect the water which will flow through the condenser tube when the apparatus is steamed out. The funnel and the collector have a common outlet into a sink. The funnel (b) carries several indentations to prevent the glass beads from leaving the distilling flask when the sample is transferred. The glass beads are used to assure steady boiling. In this manner the funnel cannot become clogged up. To prevent bumping in the steam generator a glass tube (m) is placed into the flask which is closed up in the middle

and at one end and whose open end reaches into the water (Folin and Svedberg)<sup>2</sup>

### *Procedure*

In a flask 4 ml of plasma (serum) are deproteinized with 8 ml of water and 8 ml of 20 per cent trichloroacetic acid (1) and filtered. Five ml of filtrate (corresponding to 1 ml of plasma or serum) are transferred into a micro-Kjeldahl flask and digested after the addition of 2 ml of copper sulfate-sulfuric acid (2) and 2 glass beads (to prevent bumping). The digestion is over when the residue turns colorless. If very little serum is available only 2 ml of serum may be diluted with 10 ml of water and 8 ml of trichloroacetic acid is added. For the analysis 5 ml of filtrate corresponding to 0.5 ml of

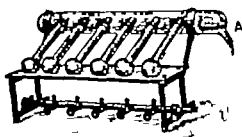


FIG 27 DIGESTION RACK

serum are used. The digestion is performed in a digestion rack (fig 27). The end (A) is connected to a water pump to remove the fumes. Suction may only be applied if no urine determinations are carried out in the same room. If for technical reasons this is unavoidable, the fumes cannot be sucked off but a rubber tubing is attached at A, which ends just above the liquid level of a water tank. Ammonia fumes represent a great danger to the accuracy of the analysis as they are drawn into the flask by the sulfuric acid during the cooling off period after digestion. To avoid this, the hot flasks are placed immediately after digestion into large glass jars with ground glass tops. Into the jars are placed a number of asbestos-lined small tin cans (food cans or Squibb ether cans, cut in half), one can for each Kjeldahl flask. In this manner the hot flasks are well protected.

The residue in the flask is diluted with water, the rim of the flask is slightly greased with vaseline the contents are transferred quanti-

tatively into the distilling flask of the apparatus and 10 ml of Kjeldahl alkali is added. Exactly 5 ml of HCl iodate solution (4) are placed into the receiver. It is distilled twice each time for 4 minutes. During the first 4 minutes the condenser tube dips into the receiver liquid later on the receiver flask is lowered to assure a free drip. After each distillation the apparatus must be washed as described above.

### *Titration*

A crystal of KI is added and the titration performed in a 10 ml semi microburette with N/100 thiosulfate solution until yellow then a few drops of starch are added and the titration continued till colorless. Simultaneously the thiosulfate value of 5 ml of HCl iodate solution is determined (blank run).

### *Calculation*

Thiosulfate used up by the blank minus thiosulfate used up by the sample multiplied by 0.14 gives the nonprotein nitrogen content of the sample in mg. If 1 ml of serum has been analyzed the non protein nitrogen content of the sample in mg per cent is obtained by multiplying the difference by 14 (see table 6).

### *Example*

thiosulfate required by the blank	9.98 ml
thiosulfate required by the sample	7.85 ml
	<hr/>
difference	2.13 ml
nitrogen content of sample	$0.14 \times 2.13 = 0.2982$ mg

Since 1 ml of serum has been analyzed the nonprotein nitrogen content of the serum is 29.82 mg per cent.

### *Colorimetric Semimicro-method for Serum and Plasma according to Folin<sup>4</sup>*

*Principle of the method* In the deproteinized filtrate the amide-bound nitrogen is converted into ammonia with concentrated  $H_2SO_4$  and after the addition of Nessler's reagent the ammonia is determined colorimetrically by comparison with a standard solution.

TABLE 6.—For the calculation of nonprotein nitrogen in mg% from the difference: (thiou/sulfate used by the sample minus thiou/sulfate used by the blank

Micro-method without distillation, see pp 144-149  
Semi-micro method with distillation see p 139

	0.00	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.50	0.55	0.60	0.65	0.70	0.75	0.80	0.85	0.90	0.95
0	—	1 56	3 11	4 67	6 23	7 78	9 33	10 89	12 44	14 00	15 55	17 11	18 67	20 23	21 78	23 33	24 88	26 44	28 00	29 55
1	—	0 70	1 10	2 10	3 50	5 50	7 50	9 50	11 50	13 50	15 50	17 50	19 50	21 50	23 50	25 50	27 50	29 50	31 50	33 50
2	31 11	32 68	34 22	35 77	37 33	38 88	40 44	42 00	43 55	45 10	46 66	48 22	49 77	51 33	52 88	54 44	56 00	57 55	59 10	60 65
3	14 00	15 10	16 10	17 20	18 30	19 40	20 50	21 60	22 70	23 80	24 90	26 00	27 10	28 20	29 30	30 40	31 50	32 60	33 70	34 80
4	28 01	29 10	30 20	31 30	32 40	33 50	34 60	35 70	36 80	37 90	39 00	40 10	41 20	42 30	43 40	44 50	45 60	46 70	47 80	48 90
5	63 01	64 10	65 20	66 30	67 40	68 50	69 60	70 70	71 80	72 90	74 00	75 10	76 20	77 30	78 40	79 50	80 60	81 70	82 80	83 90
6	28 00	29 10	30 20	31 30	32 40	33 50	34 60	35 70	36 80	37 90	39 00	40 10	41 20	42 30	43 40	44 50	45 60	46 70	47 80	48 90
7	93 31	94 40	95 50	96 60	97 70	98 80	99 90	100 00	101 10	102 20	103 30	104 40	105 50	106 60	107 70	108 80	109 90	110 00	111 10	112 20
8	42 00	43 10	44 20	45 30	46 40	47 50	48 60	49 70	50 80	51 90	53 00	54 10	55 20	56 30	57 40	58 50	59 60	60 70	61 80	62 90
9	124 4	125 5	126 6	127 7	128 8	129 9	130 0	131 1	132 2	133 3	134 4	135 5	136 6	137 7	138 8	139 9	140 0	141 1	142 2	143 3
10	42 00	43 10	44 20	45 30	46 40	47 50	48 60	49 70	50 80	51 90	53 00	54 10	55 20	56 30	57 40	58 50	59 60	60 70	61 80	62 90
11	56 00	57 10	58 20	59 30	60 40	61 50	62 60	63 70	64 80	65 90	67 00	68 10	69 20	70 30	71 40	72 50	73 60	74 70	75 80	76 90
12	135 5	136 6	137 7	138 8	139 9	140 0	141 1	142 2	143 3	144 4	145 5	146 6	147 7	148 8	149 9	150 0	151 1	152 2	153 3	154 4
13	70 00	71 10	72 20	73 30	74 40	75 50	76 60	77 70	78 80	79 90	80 00	81 10	82 20	83 30	84 40	85 50	86 60	87 70	88 80	89 90
14	168 2	169 3	170 4	171 5	172 6	173 7	174 8	175 9	176 0	177 1	178 2	179 3	180 4	181 5	182 6	183 7	184 8	185 9	186 0	187 1
15	81 00	82 10	83 20	84 30	85 40	86 50	87 60	88 70	89 80	90 90	91 00	92 10	93 20	94 30	95 40	96 50	97 60	98 70	99 80	100 90
16	717 7	718 8	719 9	720 0	721 1	722 2	723 3	724 4	725 5	726 6	727 7	728 8	729 9	730 0	731 1	732 2	733 3	734 4	735 5	736 6
17	38 00	39 10	40 20	41 30	42 40	43 50	44 60	45 70	46 80	47 90	49 00	50 10	51 20	52 30	53 40	54 50	55 60	56 70	57 80	58 90
18	248.8	249 9	250 0	251 1	252 2	253 3	254 4	255 5	256 6	257 7	258 8	259 9	260 0	261 1	262 2	263 3	264 4	265 5	266 6	267 7
19	379 9	380 0	381 1	382 2	383 3	384 4	385 5	386 6	387 7	388 8	389 9	390 0	391 1	392 2	393 3	394 4	395 5	396 6	397 7	398 8
20	126 0	126 7	127 1	128 1	128 8	129 5	130 2	130 9	131 6	132 3	133 0	133 7	134 4	135 1	135 8	136 5	137 2	137 9	138 6	139 3

est.	0.01	0.02	0.05	0.01
est.	0.31	0.03	0.03	1.24
reg.	0.11	0.23	0.12	0.06

*Reagents*

- (1) 10 per cent sodium tungstate solution\*
- (2) (a)  $2/3$  N  $H_2SO_4$  or 9.076 Gm  $KHSO_4$  in 100 ml of water  
(b) N/12  $H_2SO_4$  (1) or 1.134 Gm  $KHSO_4$  in 100 ml of water
- (3) ammonium sulfate standard solution 0.2830 Gm of ammonium sulfate (c p) (reagent) dried at  $100^\circ C$  is dissolved in water in a 1 liter volumetric flask, 1 ml. of concentrated HCl is added to prevent mold formation and the solution is made up to the mark with water. Five ml of this solution contain 0.3 mg of nitrogen
- (4) digestion mixture
  - (a) syrupy  $H_3PO_4$  (85 per cent)
  - (b) concentrated  $H_2SO_4$
  - (c) copper sulfate solution, 0.55 per cent

In a pyrex flask are mixed with caution 300 ml of syrupy phosphoric acid (85 per cent) and 100 ml of concentrated  $H_2SO_4$ . The mixture is cooled and transferred to a high 500 ml glass stoppered volumetric cylinder. Over a period of one week a precipitate of insoluble calcium compounds will form. Then 100 ml of the clear mixture is carefully added to 110 ml of 0.55 per cent copper sulfate solution. Both solutions are best kept in bottles shown in fig. 28.

- (5) Nessler's reagent (Folin)

(a) in a heavy walled flask are mixed 100 ml of water 150 Gm of KI and 110 gm of iodine. To this solution 140-150 Gm of metallic mercury are added drop by drop and with shaking over a period of 10-15 minutes. It is well shaken until the red color of the solution begins to fade. Then it is cooled with shaking under running water until the color turns green. The supernatant is decanted and the remaining mercury is washed several times with distilled water the wash water being added each time to the decanted supernatant. The liquid is made up to 2000 ml. with distilled water. The solution should be clear, otherwise it must be allowed to stand until the precipitate has settled and is again decanted.

(b) carbonate-free NaOH in a porcelain dish resting on a cork plate 500 Gm of NaOH are dissolved in 500 Gm of water with

According to a suggestion of Russel L. Haden (J Biol. Chem. 55 460 1923) the following mixture is used for deproteinizing. To one part of blood is added 8 parts of N/12  $H_2SO_4$  and 1 part of 10 per cent  $Na_2WO_4$ .

constant stirring. The solution is transferred to a bottle and the insoluble sodium carbonate is allowed to settle out. To obtain a 4 per cent solution of NaOH 55 ml. of this solution is diluted with water to 1000 ml.

*Working solution of Nessler's reagent*

150 ml. of Nessler stock solution (a)

95 ml. of carbonate free NaOH (b) made up to 1000 ml. with distilled water

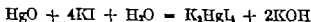
The Nessler solution may also be prepared in a simpler way as follows\* In a 1 liter volumetric flask 10.2 Gm. of dry red mercuric



FIG. 28 BOTTLE WITH DOUBLE STOPPER

oxide ( $\text{HgO}$ ) and 51 Gm. of potassium iodide crystals are mixed and shaken until the red mercuric oxide dust sticks to the KI crystals which is usually the case after 5 minutes. Then 150 ml. of distilled water is added and again shaken. The mixture is readily soluble in the cold. To this solution 100 ml. of carbonate-free 50 per cent NaOH is added and it is made up to 1000 ml. with water. An excellent Nessler reagent will result.

*Equation*



Mainly according to L. F. Wiels J. of Lab. and Clin. Med. 181 1941



With regard to the nesslerization the following must be noted digestion acid and nessler solution must be compatible, otherwise cloudiness will occur during the colorimetric reading With phenol phthalein as indicator 20 ml of N/1 HCl should require 11.0–11.5 ml of Nessler reagent for neutralization If less is used the reagent is too alkaline and must be adjusted with the following solution

(c) When working with Folin reagent 150 ml of solution 5a is made up to 1000 ml with distilled water (no NaOH is used)

When working with Wiels reagent 10.2 Gm HgO and 51 Gm of KI are dissolved in water as described above and made up to 1000 ml with water only

If 20 ml of N/1 HCl require 9 ml of Nessler reagent instead of 11 ml., the solution is too alkaline and must be adjusted according to the equation

$$\frac{11.0}{9.0} = \frac{1000}{X}, X = \frac{9.0 \times 1000}{11.0} = 818$$

Consequently 818 ml of Nessler reagent must be diluted with 182 ml. of solution c

Now the digestion acid must be compared with the Nessler reagent One ml of the digestion acid should neutralize 9.0–9.3 ml. of Nessler reagent If this is not the case the acid must be adjusted

The Nessler reagent should not be exposed to daylight and must be kept in a dark bottle It will keep for a long period and will even improve with time.

### *Procedure*

*Deproteinizing* (a) serum or plasma In an Erlenmeyer flask 1 part of serum or plasma (2–3 ml) is diluted with 7 parts of water and 1 part of a 10 per cent sodium tungstate solution (1) is added One part of  $\frac{2}{3}$  N sulfuric acid (2a) is added with shaking from a burette or pipet and shaking is continued until a metallic sound is heard The solution is filtered after 2–3 minutes through a folded filter

(b) whole blood To 1 part of whole blood (2–3 ml) in an Erlenmeyer flask is added 8 parts of N/12 H<sub>2</sub>SO<sub>4</sub> (2b) From a burette or pipet is added with shaking 1 part of 10 per cent sodium tungstate solution (1) The procedure is continued as described under (a)

**Digestion** Into a 75 ml pyrex test tube, bearing marks at 35 ml and at 50 ml are placed 5 ml of protein free serum (plasma, whole blood) filtrate and 1 ml of digestion acid (4). Into a similar flask are placed 5 ml of standard solution (3) and 1 ml of digestion acid (4). Both solutions (standard and sample) are heated over a small flame until the water has evaporated and  $\text{SO}_2$  fumes appear. Small quartz fragments or 2 glass beads or a small piece of glass wool is added to prevent bumping. The flask is now heated for only 3 minutes. After this time the solution must be colorless. It is then cooled and diluted with water to the 35 ml mark and Nessler reagent (5) is added to the 50 ml mark with cooling, and the colors are compared in the colorimeter.

#### Calculation

For the Dubosque colorimeter

$$\frac{\text{reading of standard} \times 30}{\text{reading of unknown sample}} = \text{mg. per cent nonprotein nitrogen}$$

#### Example

reading of standard	20
reading of sample	25

$$\frac{20 \times 30}{25} = 24 \text{ mg. per cent nonprotein nitrogen}$$

#### Calculation

For the Hellige colorimeter (1) If the color of the sample is less intense than the color of the standard, the following formula is used

$$\frac{100 - y}{100} \times 30 = \text{mg. per cent nonprotein nitrogen}$$

$y$  = colorimeter reading

#### Example

$$y = 20 \quad \frac{100 - 20}{100} \times 30 = 24 \text{ mg. per cent nonprotein nitrogen.}$$

(2) If the color of the sample is more intense than the color of the standard the sample solution is placed into the wedge and the stand

and solution into the cup and the following formula is used (see appendix p 379)

$$\frac{100 \times 30}{100 - y} = \text{mg. per cent nonprotein nitrogen.}$$

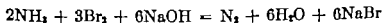
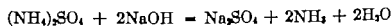
*Example*

$$y = 60, \quad \frac{100 \times 30}{100 - 60} = \frac{3000}{40} = 75 \text{ mg. per cent nonprotein nitrogen.}$$

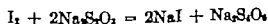
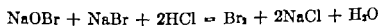
*Titrimetric Micromethod for the Determination of Nonprotein nitrogen in Whole Blood Serum or Plasma with Digestion but without Distillation.*<sup>4-6</sup>

*Principle of the method* Whole blood, serum or plasma (0.1 or 0.2 ml) is deproteinized and digested with phosphomolybdic acid as catalyst, a digestion acid being used to which a known amount of phosphoric acid is added. After the digestion the phosphomolybdic acid is converted into a complex compound with sodium fluoride and the digestion mixture is neutralized. The smallest excess of alkali during neutralization can liberate ammonia (increased temperature, shaking, etc.). But the addition of phosphoric acid and the use of a suitable indicator permit the neutralization with NaOH under formation of disodiumammonium phosphate. Under these conditions the ammonia is resistant to heat and shaking. In the digestion flask the ammonia of the sample is oxidized to nitrogen with hypobromite solution of known titer, the excess is titrated with sodium thiosulfate in the presence of KI.

*Reaction equation*



The bromine in excess of that used for the oxidation is determined iodometrically according to the following equation



It can be noted from the equation that 3 atoms of Br correspond to 1 molecule of  $\text{NH}_3$ , a small amount of ammonia will use up a relatively large amount of  $\text{Br}_2$ .

### Reagents

#### *For protein precipitation*

(1) in a 1 liter volumetric flask 20 Gm of anhydrous sodium

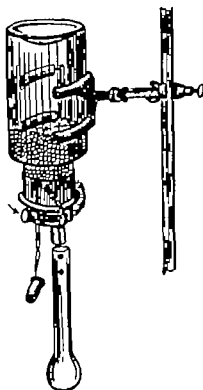


FIG 29 AUTOMATIC DISPENSER FOR GLASS BEADS

sulfate are dissolved in water 69 ml of  $\frac{1}{2}$  N  $\text{H}_2\text{SO}_4$  are added and made up to the mark with water

(2) five grams of anhydrous sodium sulfate and 8.3 gram of phosphomolybdic acid are dissolved in 200 ml. of water and 4.8 ml of 27 per cent NaOH is added. The mixture is heated over a bunsen burner for 30 minutes. After cooling 10.6 ml of  $\text{H}_2\text{SO}_4$  conc. are added and made up to 1000 ml with water

#### *For the digestion*

(3) one gram of phosphomolybdic acid is dissolved in approximately 50 ml of water. After the addition of 1 ml. of 83 per cent

ard solution into the cup and the following formula is used (see appendix, p 379)

$$\frac{100 \times 30}{100 - y} = \text{mg. per cent nonprotein nitrogen.}$$

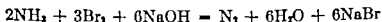
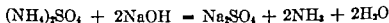
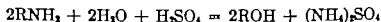
*Example*

$$y = 60, \quad \frac{100 \times 30}{100 - 60} = \frac{3000}{40} = 75 \text{ mg per cent nonprotein nitrogen.}$$

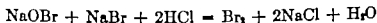
*Titrimetric Micromethod for the Determination of Nonprotein nitrogen in Whole Blood Serum or Plasma with Digestion but without Distillation.<sup>1-4</sup>*

*Principle of the method* Whole blood, serum or plasma (0.1 or 0.2 ml) is deproteinized and digested with phosphomolybdic acid as catalyst, a digestion acid being used to which a known amount of phosphoric acid is added. After the digestion the phosphomolybdic acid is converted into a complex compound with sodium fluoride and the digestion mixture is neutralized. The smallest excess of alkali during neutralization can liberate ammonia (increased temperature, shaking etc.) But the addition of phosphoric acid and the use of a suitable indicator permit the neutralization with NaOH under formation of disodiumammonium phosphate. Under these conditions the ammonia is resistant to heat and shaking. In the digestion flask the ammonia of the sample is oxidized to nitrogen with hypobromite solution of known titer, the excess is titrated with sodium thiosulfate in the presence of KI.

*Reaction equation*



The bromine in excess of that used for the oxidation is determined iodometrically according to the following equation



It can be noted from the equation that 3 atoms of Br correspond to 1 molecule of  $\text{NH}_3$ ; a small amount of ammonia will use up a relatively large amount of  $\text{Br}_2$ .

### Reagents

#### *For protein precipitation*

(1) in a 1 liter volumetric flask 20 Gm of anhydrous sodium

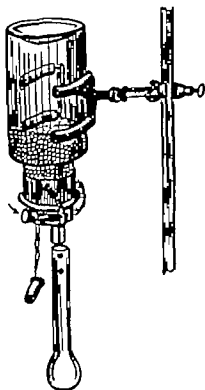


FIG 29 AUTOMATIC DISPENSER FOR GLASS BEADS

sulfate are dissolved in water 60 ml of  $\frac{1}{2}$  N  $\text{H}_2\text{SO}_4$  are added and made up to the mark with water

(2) five grams of anhydrous sodium sulfate and 8.3 gram of phosphomolybdic acid are dissolved in 200 ml of water and 4.8 ml of 27 per cent NaOH is added. The mixture is heated over a bunsen burner for 30 minutes. After cooling 10.6 ml of  $\text{H}_2\text{SO}_4$  conc. are added and made up to 1000 ml with water

#### *For the digestion*

(3) one gram of phosphomolybdic acid is dissolved in approximately 50 ml of water. After the addition of 1 ml of 33 per cent

sodium hydroxide the solution is heated for twenty minutes to eliminate any possibly present ammonia. After cooling, 30 ml of concentrated  $H_2SO_4$  and 5 ml of syrupy  $H_3PO_4$  are added and the solution is made up to 100 ml. (If no phosphoric acid resistant digestion flasks are available, no phosphoric acid can be used for the digestion. After the digestion with  $H_2SO_4$  only, a 10 per cent solution of  $Na_2HPO_4$  is added in this case.)

It is recommended to store the acid solutions (1-3) in bottles as shown in fig. 28 to prevent contamination with ammonia.

*For the neutralization*

(4) (a) 27 per cent NaOH prepared from purest grade of sodium hydroxide

(b) 15 mg of methyl red (Kahlbaum) and 60 mg of thymol blue are dissolved in 20 ml of N/1 NaOH and made up to 1000 ml. with water

(c) saturated sodium fluoride solution (approximately 5 per cent)

The working solution is prepared by mixing 3 parts of solution (a)  $\frac{1}{2}$  part of solution (b) and  $1\frac{1}{2}$  parts of solution (c). This mixture will keep indefinitely.

*For the titration*

(5) buffer solution (a) 84.5 Gm of boric acid and 14.6 Gm of NaOH (pellets) are dissolved in approximately 800 ml of water. The solution is heated for 30 minutes to eliminate any possibly present ammonia and after cooling made up to 1000 ml. (instead of this solution a 10 per cent  $Na_2HPO_4$  solution may be used)

(b) in a 1 liter volumetric flask 20 Gm of KBr are dissolved in water. 8 Gm (2.5 ml) of bromine are added and after the bromine has dissolved the solution is made up to the mark with water.

If no bromine is available the bromine solution may be prepared as follows: 32 Gm of KBr and 2.8 Gm of  $KBrO_3$  are dissolved in water in a 1 liter volumetric flask. 100 ml of N/1  $H_2SO_4$  is added and after standing for 30 minutes made up to the mark with water. The final acidity of both solutions is identical since in the latter solution 100 ml of  $H_2SO_4$  is used up to convert the bromate to bromine.

Before the determination 10 ml of bromine solution is placed into a 50 ml volumetric flask and dilute NaOH is added until the color changes from brown to a lemon yellow. Now approximately 25 ml.

of buffer solution (5a) (or 10 per cent  $\text{Na}_2\text{HPO}_4$ ) are added and made up to the mark with water. This hypobromite solution must be prepared fresh each time.

(6) KI crystals

(7) fuming  $\text{HCl}$  diluted 1:1 with water

(8) 0.25 per cent starch solution

(9) N/100 sodium thiosulfate solution (see appendix)

### Procedure

A number of test tubes equal to the number of determinations to be performed are filled with 5.8 ml of sodium sulfate solution (1) (It is recommended to use pipets bearing marks at 5.8 ml and 6 ml. If large amounts of serum are available 0.4 ml of serum is transferred to a test tube containing 11.6 ml of sodium-sulfate solution.) With a capillary pipet 0.2 ml of blood is drawn from the finger tip. The outside of the pipet is wiped and the blood blown into the sulfate solution. The pipet is rinsed several times by sucking up and blowing out of the sulfate solution. Then two ml of phosphomolybdic acid (2) are added, the contents well mixed and heated for 4-5 minutes in a water bath kept at  $50^\circ\text{C}$ . (If 0.4 ml of serum is used, 4 ml. of phosphomolybdic acid must be added.) In the same manner 2 or 3 blank determinations are performed. After cooling the solution is filtered (Whatman Nr 42 7 cm diameter; it is important to keep these filters in a glass jar with ground glass top.) Six ml of filtrate, 2 ml of digestion acid (3) and a few glass beads or some glass wool are placed into a micro-Kjeldahl flask, which has been modified for this determination as follows: length of flask 135 mm, diameter of neck of flask 20 mm, length of neck 85 mm, diameter of flask bulb 40 mm, volume approximately 35 ml. When a series of determinations is to be performed it is recommended to use the apparatus as shown in fig. 20, which automatically dispenses 2 glass beads into each flask.

The digestion is completed when the blue color has disappeared and no more sulfurous acid fumes are visible\*. After cooling the residue is diluted with 2-3 ml of water (or with 2 ml of  $\text{Na}_2\text{HPO}_4$ ).

The connection between digestion plate and evacuating tube is maintained with the aid of movable iron clamps attached to the apparatus as shown in figure 27. If urine analyses are performed in the same laboratory the same precautions have to be applied as mentioned on p. 137.



if the digestion liquid does not contain any phosphoric acid) and 5 ml of the mixed indicator NaOH-sodium fluoride solution are added drop by drop and with outside cooling. The faintly acid solution is neutralized drop by drop with 27 per cent NaOH until the color changes to blue. (Approximately the same number of drops should be required for all flasks of the same series. The 27 per cent NaOH is drawn from a burette whose stopcock is greased with graphite grease to prevent sticking. The graphite grease is prepared as follows: one part finely pulverized and sifted graphite is well mixed with 9 parts of vaseline.) Phosphoric acid is added to the digestion acid to form  $\text{Na}_2\text{NH}_4\text{PO}_4$  during neutralization. By its buffer action, the phosphoric salt hinders the sudden change from acid to alkali, thus preventing the escape of ammonia. The mixed indicator also helps to neutralize step by step so that the reaction can take place at the constant pH.

All flasks of a series of determinations may be neutralized at the same time and 5 ml of hypobromite solution (5) is also added to each flask at the same time. Immediately before titration each flask receives a few crystals of KI and 2-3 ml of HCl solution (7). The HCl is best kept in an automatic pipet, set up next to the thiosulfate burette. The liberated iodine is now titrated to yellow with N/100 sodium thiosulfate solution (9). A few drops of starch are added and the titration continued till colorless.

### Calculation

Thiosulfate used up by the blank minus thiosulfate used up by the sample multiplied by 0.0467 (atomic weight of nitrogen 0.14 divided by 3) results in the nitrogen content of the sample in mg. if N/100 thiosulfate is used. If 0.2 ml of material was analyzed it is multiplied by 31.1 to obtain the nitrogen content in mg. per cent. If 0.1 ml of material was used (0.9 ml of deproteinizing solution (1)) the factor 62.2 is applied. The factors are arrived at as follows:

$$\frac{0.0467 \times 100}{0.15} = 31.1 \quad \frac{0.0467 \times 100}{0.075} = 62.2$$

6 ml of filtrate correspond to 0.15 ml or 0.075 ml of starting material

*Example*

thiosulfate used for blank	0.80 ml
thiosulfate used for sample	8.85 ml
	<hr/>
difference	0.05 ml
	$0.05 \times 51.1 = 2.55$ mg. per cent
	nonprotein nitrogen content

For the rapid calculation see table 6

*Rapid Direct Titrimetric Determination of Nonprotein nitrogen in 0.1 ml. or 0.2 ml. of Whole Blood or Serum without Digestion or Distillation*<sup>9, 11</sup>

*Principle of the method* Whole blood, serum or plasma is deproteinized; to the filtrate is added a hypobromite solution and the excess of bromine is titrated iodometrically. With this method it is possible to determine the nonprotein nitrogen content of 0.1 or 0.2 ml. of whole blood, serum or plasma within 5-10 minutes with the same grade of accuracy as obtained by the micro Kjeldahl method, but without digestion or distillation. Under the conditions of the method not only urea and ammonia are attacked but all physiologic amino compounds are also determined. Not included are indol compounds, having the nitrogen in the ring but this is insignificant for all practical purposes since indol compounds do not normally occur in blood and in cases of uremia they rarely exceed 0.01-0.5 mg. per cent nitrogen.

*Reagents*

## (1) Deproteinizing solution\*

In a 1 liter volumetric flask 3.48 Gm. of sodium tungstate or 44.8 ml. of a 10 per cent sodium tungstate solution, 2 Gm. of sodium citrate, 6.4 Gm. of sodium sulfate are dissolved in approximately 500 ml. of water, 11.8 ml. of  $N/1$   $H_2SO_4$  or 67.2 ml. of  $\frac{1}{3}$   $N$   $H_2SO_4$ † and 2 Gm. of cadmium sulfate are added and all is made up to the mark with water. It is important to maintain the correct sequence of adding the reagent in order to avoid the formation of precipitates.

<sup>9</sup> Modified Abrahamson reagent (Abrahamson & reports in *Clinical Chemistry* by Frank Weleher, London (C. Springer & Hall).

† Instead of 67.2 ml. of  $\frac{1}{3}$   $N$   $H_2SO_4$ , 0.2 gm. of  $H_2SO_4$  can be used.

## (2) Hypobromite solution

## (I) boric acid solution

(a) 84.5 Gm of boric acid (pure) and 14.0 Gm NaOH are dissolved in approximately 800 ml of water, boiled for 30 minutes to eliminate all ammonia and after cooling made up to 1000 ml

(b) saturated sodium fluoride solution (approximately 5 per cent)

## (c) 27 per cent NaOH

250 ml of boric acid solution (a), 150 ml of saturated sodium fluoride solution (b) and 50 ml of 27 per cent NaOH (c) are mixed (5:3:1). This mixture will keep well.

## (II) bromine solution

In a 1 liter volumetric flask 20 Gm of KBr is dissolved in approximately 50 ml of distilled water, 8 Gm. of pure bromine (2.5 ml.) is added, it is shaken until the bromine has dissolved and made up to the mark with water

Immediately before use 1 part of solution II (bromine solution) is added to 9 parts of mixture Ia-c (boric acid, sodium fluoride, NaOH). Five ml of this hypobromite solution should use up approximately 10 ml of N/200 sodium thiosulfate solution

## (3) N/200 thiosulfate

## (4) KI crystals

## (5) 0.25 per cent starch solution

## (6) 18 per cent HCl (fuming HCl diluted 1:1 with water)

Cadmium sulfate is added to the deproteinizing solution to prevent the appearance of hydrogen sulfide compounds in the filtrate, especially when working with whole blood. These compounds require large amounts of bromine (1 H<sub>2</sub>S requires 4 Br). Cadmium sulfate serves to precipitate the organic SH-compounds.

The hypobromite solution contains boric acid to prevent the reducing effect of sugar by forming a complex compound. This is not necessary in the original Kjeldahl method since sugar is destroyed during the digestion. It has been proved that in the presence of boric acid bromine will not affect even large amounts of sugar.

*Procedure*

Short test tubes are filled with 5.04 ml \* or 10.08 ml. of depro-

\* A 5.04 ml pipet can be improvised by making a new mark at 5.04 ml on a 5 ml pipet.

using solution (1) (This solution can be kept in storage in capped test tubes) With a capillary pipet 0.1 ml. or 0.2 ml. of serum, plasma, or whole blood (from the finger tip) is transferred to the solution and the pipet is rinsed by sucking the solution up and blowing it out several times. The suspension is centrifuged and filtered after standing for five minutes. Four ml. of clear supernatant or filtrate are measured out into each of two wide test tubes, 4 ml. of hypobromite solution (2) are added to each sample, well shaken and allowed to stand for 2-3 minutes. Several crystals of sodium and 2-3 ml. of HCl (6) are added and the titration performed with N/200 thiosulfate till yellow, consequently a few drops of starch solution (5) are added and the titration continued till colorless. A blank determination is carried out using 4 ml. of deproteinizing solution (1) and 5 ml. of hypobromite solution (2).

#### *Calculation*

The amount of thiosulfate used for the sample (V) is subtracted from the amount used for the blank (L), the difference is multiplied by 2333 (atomic weight of nitrogen 0.14 divided by 6) and divided by 0.0778 (0.0778 ml. = amount of unknown sample). Multiplication by 100 gives the amount of nitrogen in the sample in mg. per cent (when N/200 thiosulfate is used)

$$\frac{5.14}{4} = \frac{0.1}{X}, X = 0.0778 \quad \frac{0.0233 \times 100}{0.0778} = 30$$

$$(L - V) \times 30 = \text{mg per cent nitrogen}$$

#### *Example*

$$L = 9.85$$

$$V = 7.53$$

$$\text{difference} = 2.32 \quad 2.32 \times 30 = 69.6 \text{ mg per cent nitrogen}$$

#### *Gravimetric Direct Determination of Nonprotein Nitrogen in 0.05 or 0.01 ml. of Whole Blood, Serum, or Plasma without Digestion or Distillation<sup>13</sup>*

*Principle of the method* See method 4 p. 149

*Reagents* See method 4 p. 149

A N/1000 thiosulfate solution is used instead of N/200 thiosulfate

## (2) Hypobromite solution

## (I) boric acid solution

(a) 84.5 Gm. of boric acid (pure) and 14.0 Gm. NaOH are dissolved in approximately 800 ml. of water, boiled for 30 minutes to eliminate all ammonia and after cooling made up to 1000 ml.

(b) saturated sodium fluoride solution (approximately 5 per cent)

## (c) 27 per cent NaOH

250 ml. of boric acid solution (a), 150 ml. of saturated sodium fluoride solution (b) and 50 ml. of 27 per cent NaOH (c) are mixed (5:3:1). This mixture will keep well.

## (II) bromine solution

In a 1 liter volumetric flask 20 Gm. of KBr is dissolved in approximately 50 ml. of distilled water, 8 Gm. of pure bromine (2.5 ml.) is added, it is shaken until the bromine has dissolved and made up to the mark with water.

Immediately before use 1 part of solution II (bromine solution) is added to 9 parts of mixture Ia-c (boric acid, sodium fluoride, NaOH). Five ml. of this hypobromite solution should use up approximately 10 ml. of N/200 sodium thiosulfate solution.

## (3) N/200 thiosulfate

## (4) KI crystals

## (5) 0.25 per cent starch solution

## (6) 18 per cent HCl (fuming HCl diluted 1:1 with water)

Cadmium sulfate is added to the deproteinizing solution to prevent the appearance of hydrogen sulfide compounds in the filtrate, especially when working with whole blood. These compounds require large amounts of bromine (1 H<sub>2</sub>S requires 4 Br). Cadmium sulfate serves to precipitate the organic SH-compounds.

The hypobromite solution contains boric acid to prevent the reducing effect of sugar by forming a complex compound. This is not necessary in the original Kjeldahl method since sugar is destroyed during the digestion. It has been proved that in the presence of boric acid bromine will not affect even large amounts of sugar.

*Procedure*

Short test tubes are filled with 5.04 ml.\* or 10.08 ml. of depro-

\* A 5.04 ml. pipet can be improvised by making a new mark at 5.04 ml. on a 5 ml. pipet.

teinizing solution (1) (This solution can be kept in storage in stoppered test tubes.) With a capillary pipet 0.1 ml or 0.2 ml of serum plasma, or whole blood (from the finger tip) is transferred into the solution and the pipet is rinsed by sucking the solution up and blowing it out several times. The suspension is centrifuged or filtered after standing for five minutes. Four ml of clear supernatant or filtrate are measured out into each of two wide test tubes, 5 ml of hypobromite solution (2) are added to each sample, well shaken and allowed to stand for 2-3 minutes. Several crystals of KI and 2-3 ml. of HCl (6) are added and the titration performed with N/200 thiosulfate till yellow consequently a few drops of starch solution (5) are added and the titration continued till colorless.

A blank determination is carried out using 4 ml. of deproteinizing solution (1) and 5 ml of hypobromite solution (2)

### Calculation

Amount of thiosulfate used for the sample (V) is subtracted from the amount used for the blank (L), the difference is multiplied by 0.02333 (atomic weight of nitrogen 0.14 divided by 6) and divided by 0.0778 (0.0778 ml = amount of unknown sample). Multiplication by 100 gives the amount of nitrogen in the sample in mg per cent (when N/200 thiosulfate is used)

$$\frac{5.14}{4} = \frac{0.1}{X}, X = 0.0778 \quad \frac{0.0233 \times 100}{0.0778} = 30$$

$$(L - V) \times 30 = \text{mg per cent nitrogen}$$

### Example

$$L = 9.85$$

$$V = 7.53$$

$$\text{differ} = 2.32 \quad 2.32 \times 30 = 69.6 \text{ mg per cent nitrogen}$$

### *Titrimetric Direct Determination of Nonprotein-Nitrogen in 0.02 or 0.01 ml of Whole Blood, Serum, or Plasma without Digestion or Distillation<sup>13</sup>*

*Principle of the method* See method 4 p 149

*Reagents.* See method 4 p 140

A N/1000 thiosulfate solution is used instead of N/200 thiosulfate

(3) It is prepared each time before use by diluting the N/200 solution 5 times

The hypobromite solution (2) is prepared by adding  $\frac{1}{2}$  part of solution II (bromine solution) to 9 parts of the mixture Ia-c, 5 ml of this hypobromite solution should require approximately 10 ml. of N/1000 thiosulfate solution

### *Procedure*

Short test tubes are filled with 4 ml of deproteinizing solution (1) each (This solution can be kept in storage in stoppered test tubes) With a capillary pipet\* 0.02 or 0.01 ml of serum, plasma, or whole blood is transferred into the solution and the pipet is rinsed repeatedly by sucking up and blowing out of the mixture After a few minutes the tubes are centrifuged Kjeldahl flasks or any other long necked flasks have been filled with 5 ml of hypobromite solution Each flask receives 3 ml of clear supernatant and 2 flasks serving as blanks receive 3 ml of deproteinizing solution (1) The flasks are closed with clean dust-free corks and should only be opened immediately before titration For the remainder of the procedure (see method 4 p 149)

### *Calculation*

Amount of thiosulfate required for the blank (L) minus thiosulfate required for the sample (V) multiplied by 0.00467 (atomic weight of nitrogen 0.0143) divided by 0.01492 (0.01492 ml = amount of analyzed material) and multiplied by 100 results in the nitrogen content of the sample expressed in mg per cent, provided N/1000 thiosulfate has been used

$$\frac{4.02}{3} - \frac{0.02}{X} \quad X = 0.01492 \quad \frac{0.00467 \times 100}{0.01492} = 31.3$$

$$(L - V) \times 31.3 = \text{mg per cent nitrogen}$$

### *Example*

$$L = 8.37$$

$$V = 7.10$$

$$\text{difference } 1.27 \quad 1.27 \times 31.3 = 39.75 \text{ mg per cent nitrogen}$$

See append p 361

The normal value for nonprotein nitrogen is 25-35 mg per cent. It is increased in the following diseases:

(1) chronic nephritis mainly in the last stages of glomerulonephritis or during acute recurrences. In these cases nonprotein nitrogen values of 300 mg per cent and more have been encountered.

(2) in all diseases accompanied by oliguria and anuria: acute nephritis, sublimite or other mercuric poisoning, bilateral occlusion of the urethra or unilateral occlusion with reflexory anuria, chronic heart disease with congestion and tendency toward edema formation, postoperative anuria, etc.

(3) diseases accompanied by destruction of kidney tissue: tuberculosis of the kidney, cystic kidney, pyonephrosis, hydronephrosis.

(4) hypertrophy of the prostate.

(5) acute intestinal occlusion.

(6) extensive loss of fluid: cholera asiatica, cholera nostras, dysentery, vomiting, etc.

(7) slight rise in gout and myeloid leucemia as sign of increased uric acid.

#### DETERMINATION OF UREA AND UREA CLEARANCE TEST

##### *Titrimetric Determination in Whole Blood (Hemolyzed), Serum and Plasma with Distillation<sup>11, 12</sup>*

*Principle of the method:* Urea is converted into ammonia by urease. The urease together with the proteins are precipitated by sodium tungstate and sulfuric acid; the ammonia is distilled in the apparatus of Parnas-Wagner (fig. 26) and determined titrimetrically.

##### *Reagents:*

(1) acetate buffer<sup>14</sup>: 3.0 ml. of a 1.0 per cent sodium acetate solution containing 1 ml. of glacial acetic acid per 100 ml. are made up to 100 ml. with distilled water.

(2) 0.1 *Cm.* urease: 1 tablet of urease (Squibb) is finely powdered and suspended in 10 ml. of water.

(3) deproteinizing solution: immediately before use 10 ml. of a 10 per cent sodium tungstate solution are mixed with 12 ml. of 3 *N*  $\text{H}_2\text{SO}_4$  and made up to 100 ml. with water.

(4) sodium tetraborate: 6 *Cm.* of sodium tetraborate, 1 *Cm.* of sodium tartrate, 2 ml. of a 2.0 per cent copper sulfate solution and



4 ml of N/1 NaOH are dissolved in distilled water and made up to 100 ml

(5-9) sodium hypobromite, potassium iodide hydrochloric acid, starch and sodium thiosulfate (see determination of nonprotein-nitrogen, p 140)

### *Procedure*

The required number of test tubes are filled with 4.8 ml each of the acetate buffer solution (1) (The pipets should bear marks at 4.8 ml and 5 ml) With a capillary pipet 0.2 ml of blood is drawn from the ear lobe or finger tip, the pipet is wiped carefully on the outside the contents are delivered into the tubes and the pipets are rinsed twice by sucking up and blowing out of the mixture For the blank run ( $\text{NH}_3$  content of the reagents) 2 or 3 test tubes are filled with 5 ml. of the buffer solution (1) and treated like the unknown sample Urease suspension is added (2), the tubes are closed with clean cork stoppers and placed into an incubator of exactly  $37^\circ \text{C}$  for 40-45 minutes. At the end of this period the tubes are removed, 6 ml of sodium tungstate-sulfuric acid solution (3) are added to each one. The tubes are now placed into a water bath of approximately  $40^\circ \text{C}$  for 4-5 minutes. After cooling the precipitate is filtered off (Whatman Nr 42, 7 cm diameter) Now 9 ml of filtrate are transferred into the Parnas-Wagner distillation apparatus, the funnel is washed with 6 ml of tetraborate solution, and the liberated  $\text{NH}_3$  distilled twice, each time over a period of four minutes, care being taken that during the first four minute period the condenser tube reaches well below the surface of the liquid in the receiver flask. The receiver flask contains 5 ml of N/50 sodium hypobromite solution. (If small normal amounts of urea are expected and also in research work it is recommended to use less hypobromite, 2 ml. of hypobromite for 45 mg per cent of nitrogen.

### *Titration*

Directly before titration each flask receives a crystal of KI and approximately 2 ml of HCl The liberated iodine is titrated with N/200 sodium thiosulfate to yellow and towards the end with a few drops of starch solution to colorless.

*Calculation*

Thiosulfate (N/100) used up by the blank minus thiosulfate used up by the sample multiplied by 31.1 results in the urea nitrogen content of the sample in mg per cent. If N/200 thiosulfate is used the difference is multiplied by 15.5.

*Example*

Thiosulfate required by the blank	5.02 ml
thiosulfate required by the sample	5.01 ml
difference	0.01 ml

$$0.01 \times 31.1 = 0.31 \text{ mg per cent urea nitrogen}$$

direct calculation see table 6

If the above obtained value for nitrogen is multiplied by 2.14 (or the thiosulfate difference by 60.0) the result is expressed as mg per cent urea.

$$\frac{60 \text{ (molecular weight of urea)}}{2 \times 14 \text{ (molecular weight of nitrogen)}} = 2.14$$

### *Titrimetric Determination of Urea in Nonhemolyzed Blood with Distillation*

Urea (or urea nitrogen) may also be determined in a nonhemolyzed blood sample just as the nonprotein nitrogen. When hemolyzed blood or serum is used for the determination an aqueous acetate buffer is employed, whereas in the below described method the blood is added to a buffered hypertonic sodium sulfate solution and exposed to the effect of the urease at incubator temperature.

*Reagents*

(1) In a 100 ml volumetric flask 20 ml of a 10 per cent solution of anhydrous sodium sulfate are mixed with 3.5 ml of a 15 per cent sodium acetate solution containing 1 ml of glacial acetic acid per 100 ml of solution and made up to the mark.

(2) 0.1 Gm of urease is finely powdered and suspended in 10 ml of a 2 per cent sodium sulfate solution.

(3) deproteinizing solution Immediately before use equal parts of 10 per cent sodium tungstate solution and  $\frac{1}{2}$  N  $H_2SO_4$ \* are mixed. All other reagents see determination of nonprotein nitrogen (p 146)

### *Procedure*

The required number of test tubes according to the number of analyses to be performed, is filled with 4.8 ml of hypertonic buffer solution (1). With a capillary pipet 0.2 ml of blood is drawn from the finger tip and after the outside of the pipet has been wiped clean, the contents are blown into the test tube and the pipet is rinsed as usual. For the blank run 2 or 3 test tubes are filled with 5 ml of buffer solution (1) and treated as described for the sample. One ml of urease suspension is added, the tubes are closed with clean corks and left in an incubator at 37° C for 45 minutes. Then they are allowed to cool down and 2 ml of sodium tungstate-sulfuric acid are added drop by drop with shaking. The tubes are left at room temperature for fifteen minutes and then centrifuged at high speed. 6 ml of the clear supernatant (filter if necessary) are transferred into the Parnas-Wagner apparatus and made alkaline with 6 ml of copper borate solution. The remainder of the procedure as well as the calculation are performed as described for hemolyzed blood (serum).

### *Titrimetric Determination of Urea without Distillation (with Static Distillation)<sup>17</sup>*

*Principle of the method* Urea is converted into an ammonia salt by urease. The ammonia is liberated by the addition of a concentrated potassium carbonate solution and is absorbed in 40 per cent sulfuric acid. The excess sulfuric acid is neutralized and to the neutralization product a measured amount of hypobromite solution of known titer is added. After shaking the excess hypobromite is determined iodometrically.

*Reaction equation* (see p 144)

### *Apparatus*

Alcohol flasks according to Widmark are used (fig 30) where a second smaller dish (c) is placed into the dish (b). Before new flasks

\* Instead of  $\frac{1}{2}$  N  $H_2SO_4$  a solution of 9.076 Gm  $KHSO_4$  in 100 ml of water may be used.

are used for the first time they should be washed carefully with concentrated nitric acid and water otherwise it suffices to wash with water. The flasks must be absolutely dry, the ground glass parts are greased with pure vaseline. For cleaning the vaseline is removed with dry gauze and the ground glass rinsed with hot water.

### Reagents

(1) urease suspension

(a) buffer solution 31.5 Gm of sodium acetate 2 Gm of sodium oxalate 1 Gm of saponin (purest grade, Merck) and 2 l ml of glacial

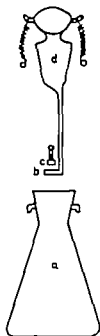


FIG. 30 Apparatus for the determination of urea without distillation

acetic acid are dissolved in 1 liter of water. If only serum or plasma is to be used saponin and oxalate may be omitted.

(b) urease tablets Squibb (1 tablet = 0.1 Gm.) Before use one tablet is pulverized and suspended in 10 ml of buffer solution.

(2) to 12.5 ml of distilled water is added carefully 10 ml of  $\text{H}_2\text{SO}_4$  concentrated and 2.5 ml of concentrated syrupy  $\text{H}_3\text{PO}_4$ .

(3) potassium carbonate solution 50 Gm of anhydrous potassium carbonate are dissolved in 50 ml of redistilled water.

(4) 15 mg of methyl red and 75 mg of thymol blue are dissolved in 10 ml. of N/1 NaOH and made up to 100 ml with water.

(5) 70 ml of N/1 NaOH is mixed with 5 ml of indicator solution (4) and made up to 100 ml. with distilled water

Test for correct ratio between solutions (2) and (5) When 5 ml of alkali solution (5) is mixed with 0.25 ml of acid (2) a faint pink color should result If the color is bright red, the NaOH content of the mixture must be increased, if a yellow or blue color appears the NaOH solution must be diluted

(6) (a) bromine solution in a 1000 ml volumetric flask 20 Gm. of KBr are dissolved in approximately 100 ml. of water, 2.5 ml of bromine is added and after the bromine has dissolved, the solution is made up to the mark with water

(b) buffer solution 84.5 Gm of boric acid crystals (not pulverized or molten) and 15.6 Gm NaOH are dissolved in approximately 800 ml of water, heated for thirty minutes to eliminate any possibly present ammonia and, after cooling is made up to 1000 ml. Before use, 10 ml of the bromine solution (a) is mixed in a 50 ml. volumetric flask with 25 ml of buffer solution (b) and is made up to the mark with water

(7) fuming HCl, diluted with water 1:1

(8) 0.25 per cent starch solution

(9) KI

(10) N/100 sodium thiosulfate solution

### *Procedure*

The required number of flasks (fig. 30)—2 for each analysis, and 2 blank runs are filled with 0.8 ml of urease suspension The sides of the flasks must be free from urease suspension With a capillary pipet 0.1 or 0.2 ml of blood (serum or plasma) is added to the urease and the pipet is rinsed several times as usual The glass stopper is replaced immediately, without greasing Samples and blank determinations are placed into an incubator at 37° C for forty five minutes. After that time the contents are well mixed cooled to room temperature and the ground glass stopper is greased with vaseline The small dishes (c) are filled with 0.25 ml of acid mixture (2) and placed upon the larger dishes (b) which are attached to the drawn-out part of the glass stopper From a fast-flowing pipet 0.5 ml of potassium carbonate solution (3) is delivered into the flask, the stopper is put on and fastened with springs. Now the flasks are kept for 55–60 minutes (not longer) at 50° C or for three hours

at 37° C in an incubator. They may also be left for 14–16 hours at room temperature. After this time they are removed from the incubator, allowed to cool down and the stopper is carefully removed. The dish containing the acid mixture is lifted up by gripping the stem with a pair of forceps and it is slipped carefully into a Hagedorn-Jensen tube containing 5 ml of indicator NaOH mixture (5). Now the samples are neutralised with N/1 NaOH and 5 ml of hypobromite are added from a pipet with a capillary tip. A few KI crystals and 2 ml of HCl are added and the liberated iodine titrated with N/100 sodium thiosulfate from a semi microburette until yellow and after the addition of a few drops of starch the titration is continued till colorless.

### Calculation

Thiosulfate used up by the blank minus thiosulfate used up by the sample multiplied by 0.0466 (atomic weight of nitrogen 14 divided by  $3 \times 100$ ) gives urea nitrogen content of the sample in mg. If the difference is multiplied by 23.3 the urea nitrogen content in mg per cent is obtained when 0.2 ml of blood are used. For 0.1 ml. of blood the factor 46.6 is applied.

### Example

thiosulfate required by the blank	6.63 ml
thiosulfate required by the sample	5.33 ml
difference	1.28 ml
	$1.28 \times 23.3 = 29.82$

mg. per cent urea nitrogen. See also table 10 p 220. The table may also be used to calculate the urea nitrogen content when working with the above described method, in this case the figures must be divided by 10.

If the nitrogen value is multiplied by 2.14 or the thiosulfate difference by 49.86 the urea content in mg per cent is obtained.

### Colorimetric Determination of Urea in Whole Blood Serum or Plasma (0.2 ml Sample)

*Principle of the method* Urea is converted into ammonia by urease and the ammonia is determined colorimetrically with Nessler's reagent.

*Reagents*

(1) buffer solution 31.5 Gm. of sodium acetate, 2.0 Gm. of sodium oxalate, 1.0 Gm. of saponine (purest grade, Merck) and 2.1 Gm. of glacial acetic acid are dissolved in 1 liter of water. If serum or plasma is to be analyzed oxalate and saponin may be omitted.

(2) standard solution

(a) stock solution In a 1000 ml volumetric flask 13.2 Gm. of ammonium sulfate is dissolved in distilled water and made up to the mark with distilled water. This solution corresponds to a 600 mg per cent urea solution.

(b) working standard 10 ml. of stock solution is placed into a 100 ml volumetric flask, 50 ml of glycerol is added and made up to the mark with distilled water. This solution will keep indefinitely when stored in the refrigerator.

(3) deproteinizing solution 53 ml of 10 per cent sodium tungstate solution, 400 ml of distilled water, and 0.4 ml of N/1  $\text{H}_2\text{SO}_4$  (or 13.616 Gm of  $\text{KH}_2\text{SO}_4$ ) dissolved in 100 ml of water) are placed into a 1000 ml volumetric flask. Water is then added up to a few cm below the mark. To this mixture are added 2 Gm of secondary sodium phosphate and all made up to the mark with water. The solution is stable.

(4) urease

(a) urease tablets

(b) Fifty ml of N/100  $\text{H}_2\text{SO}_4$  are added to 30 Gm of Jack bean meal and shaken for 30 minutes. Then 150 ml of glycerol are added shaken again and left standing over night. After that time the solution is filtered through linen or several layers of gauze. This solution will keep indefinitely when stored under refrigeration.<sup>11</sup>

(5) Nessler's reagent, see p. 140

*Procedure*

The amount of 0.8 ml of buffer solution (1) is placed into each of 2 round bottom centrifuge tubes. To one tube is added 0.2 ml of serum, plasma or whole blood using a capillary pipet. The pipet is rinsed by sucking and blowing out of the mixture. To the second tube is added 0.2 ml of standard solution (2). To both tubes are now added either a few grains of urease (4a) or 0.5 ml of urease

emulsion (4b) and the tubes are placed into a water bath of 45° C for twenty minutes. After this time 9 ml of deproteinizing solution (3) are added to each tube. When solid urease (4a) is used the sample is heated in a water bath of 50° C for several minutes and then filtered into a tube marked at 7 ml, until the filtrate reaches the mark. The filter paper must be chemically pure otherwise a cloudiness will appear upon addition of Nessler's reagent. When solid urease (4a) is used filtration is absolutely necessary, since centrifuging will not result in a clear supernatant. If urease emulsion (4b) is employed it is centrifuged for five minutes at 2000 r.p.m., 7 ml of supernatant are transferred to a tube marked at 7 ml. One milliliter of Nessler's reagent (5) is now added to each tube and the colors are compared in the Hellige colorimeter.

#### Calculation

$$\frac{(100 - y) \times 60}{100} = \text{mg. per cent urease} \quad y = \text{colorimeter reading.}$$

If the color of the unknown sample is more intense than the color of the standard the sample is placed into the wedge and the standard is placed into the cup and the calculation is done as follows:

$$\frac{100 \times 60}{100 - y} = \text{mg. per cent urease} \quad y = \text{colorimeter reading.}$$

#### Colorimetric Determination of Urea in 0.01-0.02 ml of Whole Blood or Serum<sup>19</sup>

*Principle of the method* Urea is converted into ammonia by urease and the ammonia is determined colorimetrically with Nessler's reagent.

#### Reagents

(1) buffer solution 31.5 Gm of sodium acetate 2 Gm of sodium oxalate 1 Gm of saponin (pure Merck) and 2 l Gm of glacial acetic acid are dissolved in 1 liter of water

(2) standard solution

(a) stock solution 13.2 Gm of ammonium sulfate are dissolved in distilled water in a 1000 ml volumetric flask and made up to the



mark with distilled water. This solution corresponds to a 600 mg per cent urea solution.

(b) working standard. 10 ml of stock solution are introduced into a 100 ml volumetric flask, 50 ml of glycerol are added and made up to the mark with distilled water. This solution will keep indefinitely when stored on ice.

(3) deproteinizing solution. into a 100 ml. volumetric flask are placed 10.5 ml of 10 per cent sodium tungstate solution and about 50 ml of distilled water, followed by 23 ml of N/1  $H_2SO_4$  and made up to the mark with distilled water. To stabilize this sodium tungstate-sulfuric acid solution 0.4 Gm of secondary sodium phosphate ( $Na_2HPO_4$ ) is dissolved in the mixture.

(4) urease suspension. 30 Gm of Jack bean meal are mixed with 50 ml of N/1000  $H_2SO_4$  and gently shaken for thirty minutes. Then 150 ml of glycerol are added, well shaken and left standing over night. After that time the solution is filtered through linen or several layers of gauze. It should be stored under refrigeration and will keep indefinitely.

(5) Nessler's reagent, see above.

### *Procedure*

Two-tenths (0.2) of a milliliter of urease-suspension (4) is added to 6 ml of buffer solution (1) and 0.48 ml. of this mixture is transferred to a round bottom centrifuge tube. From the fingertip or the earlobe 0.02 ml of whole blood (or 0.01 ml if a high value is expected) is drawn with a Sahli pipet\* and transferred into the buffer-urease mixture. The pipet is rinsed as usual. 4.8 ml of buffer-urease mixture and 0.2 ml of standard solution (2b) are placed into an identical centrifuge tube. Both tubes are placed into a water bath of 45° C for twenty minutes. To the tube containing the sample is now added 0.5 ml of deproteinizing solution (3) and to the tube containing the standard are added 5 ml of the deproteinizing solution (3). The tubes are centrifuged for five minutes at 2000 r.p.m. 0.7 ml of the supernatant from the sample is transferred into a test tube and the supernatant of the standard is poured into a tube marked at 7 ml. Now the sample tube receives 0.1 ml. of

\* Capillary pipet see appendix p. 301

Nessler's reagent and the colors are compared in the Hellige colorimeter

### Calculation

$$\frac{100 - y}{100} \times 60 = \text{mg. per cent urea} \quad y = \text{colorimeter reading.}$$

Should the color of the sample be more intense than that of the standard, the sample is diluted with distilled water, the dilution taken into consideration and calculated accordingly

If a high urea value is expected, only 0.01 ml. of blood is used 0.49 ml. of buffer urease mixture are employed in this case and the final calculated value is multiplied by 2

Under normal conditions the urea nitrogen content is 10-15 mg per cent (fasting value, 12-16 hours after last meal)

The urea level in blood depends upon the protein intake. Therefore urea values can only be compared under similar dietary conditions.

An increase is noted in all cases where an increase of nonprotein nitrogen is encountered

A decrease of urea is found

- (1) occasionally during lipid nephrosis (7-10 mg per cent u. n.)
  - (2) during acute liver insufficiency values of 5-10 U N mg per cent are found in acute yellow atrophy of the liver acute toxic liver diseases (phosphorous, arsenic, carbon tetrachloride poisoning)
- In chronic diseases (cirrhosis, congestion, tumors) a low level is rarely found.

### UREA CLEARANCE TEST<sup>21-23</sup>

*Principle of the method* The blood urea clearance test introduced by Moeller McIntosh and D. D. van Slyke may be considered as a refinement of the kidney function test. It is based on the principle that under certain conditions the rate of urea excretion is dependent on the blood urea content.

The urea clearance is an expression of the number of ml. of blood, from which urea is removed or cleared in one minute by the kidneys. This is usually expressed in terms of percentage of the average normal. It depends on the volume of urine per minute and on the body surface.

area. But only in the case of children and over- and undersized adults has the latter to be taken into account.

### *Procedure*

The following preparations and analyses are necessary

- (1) preparation of the patient
- (2) urea determination in blood
- (3) urea determination in urine
- (4) calculation and evaluation of results

#### (1) Preparation of the patient

The patient is allowed to eat an ordinary breakfast and is given an additional glass of water.\*

The bladder is immediately emptied completely, the specimen being discarded. The exact time is recorded at which the subject finishes voiding, which marks the beginning of the period 1.

At the end of approximately one hour the bladder is again completely emptied and the time noted exactly. This marks the end of period 1. The volume of the entire specimen is carefully measured and it is preserved in the refrigerator.

The patient is given another glass of water and blood is withdrawn for urea determination.

At the end of approximately two hours from the beginning of period 1 the bladder is again completely emptied and the time noted exactly as marking the end of period 2. The volume of urine is carefully measured and it is preserved in the refrigerator unless examined immediately.

Common sources of error: (A) inaccurate timing of test periods. (B) inaccurate measurement of urine volume. (C) incomplete evacuation of bladder. In cases in which interference with complete emptying is suspected (e.g. prostatic hypertrophy, cystocele, pregnancy, cervical tumors, etc.) the bladder must be emptied by catheterization. All diuretics should be avoided previous to the test.

The test is usually performed in the morning, as excretion is less liable to fluctuate in the hours between breakfast and lunch, i.e. between 9 and 12 a.m. (McHav) but if necessary it may be done at some other time at the convenience of the patient and laboratory. The patient is routinely given two glasses of water, one at the beginning of the test and one an hour later. This promotes a fairly free flow of urine during the test and diminishes the relative error that may be caused by urine retention in the bladder at the voidings.

(2) Urea determination in blood (see p 153 ff)

(3) Urea determination in urine

There are three methods available to determine urea in urine

(A) Manometric determination of urea in urine according to van Slyke

(B) Volumetric determination of urea in urine according to Kovarski

(C) Titrimetric determination of urea after decomposition by urease

(A) *Manometric determination of urea nitrogen*

### *Principle*

The urine is treated with an alkaline hypobromite solution and the nitrogen liberated from the urea is measured by the pressure which it exerts on a column of mercury when compressed to a definite volume

### *Reagents*

(1) Bromine solution 60 Gm of potassium bromide dissolved in 100 ml of water and 2.5 ml of bromine are added This solution keeps well

(2) 40 per cent sodium hydroxide

Immediately before use 1.25 ml of (1) are mixed with 0.75 ml of (2) Of this solution 1.5 ml is used for each determination

### *Procedure*

One milliliter of concentrated urine (sp gr over 1.030) or 2 ml of more dilute urine is placed in a 100 ml Erlenmeyer flask and either 19 or 18 ml of water are added to make the volume up to 20 ml \* Three Gm of permutit is added and the mixture is shaken for about four minutes to remove ammonia and filtered through a dry filter into a dry flask

One milliliter of water is placed in the cup of the van Slyke-Neill apparatus (see p 36) Two ml of the urine filtrate are pipeted through it into the reaction chamber The pipet is withdrawn the water is run into the chamber and is followed by 1.5 ml of the hypobromite solution The cock is sealed with a drop of mercury, the mercury in the chamber is immediately lowered to the 50 ml

If the urine contains albumin it should be removed as follows 2 ml of urine 12 ml of dist water 2 ml of 4.5 per cent  $ZnSO_4$  and 4 ml of  $\sim 10$   $\backslash NaOH$  are mixed together and filtered

mark and the chamber shaken for two minutes. The volume of liberated gas is then reduced to two ml and the pressure  $P_1$  is read on the manometer.

A blank analysis is run in which 2 ml of water previously shaken with permittit replaces the urine filtrate. The manometer reading is taken as  $P_0$ .

TABLE 7 — Factors by which pressures are multiplied to give urea content of urine

Temperature (°C)	Giving mg. urea per 100 cc.	
	0.1	0.2
15	6.09	3.35
16	6.07	3.34
17	6.64	3.32
18	6.02	3.31
19	6.00	3.30
20	6.57	3.29
21	6.55	3.28
22	6.53	3.27
23	6.50	3.25
24	6.48	3.24
25	6.46	3.23
26	6.44	3.22
27	6.41	3.21
28	6.39	3.20
29	6.37	3.19
30	6.35	3.18
31	6.32	3.16
32	6.30	3.15
33	6.28	3.14
34	6.26	3.13

Note the temperature of the water jacket surrounding the reaction chamber.

#### Calculation

The pressure due to the nitrogen liberated from the urea is equal to  $P_1 - P_0$ . The per cent of urea is calculated by multiplying this result by the factor given in table 7. This factor varies with the temperature.

The chamber of the apparatus need not be washed between the

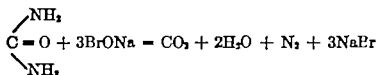
successive analyses of a series. Consequently analyses can be run off at the rate of about one every four or five minutes.

One blank analysis run at the beginning, serves for an entire series of analyses.

This method is convenient for the occasional analysis since no standards are required (with consequent danger of deterioration on long standing) and the reagent keeps indefinitely

*(B) Volumetric determination of urea in urine<sup>22</sup> (according to Kowarski)*

*Principle of the method* Urea is decomposed into nitrogen, water and  $\text{CO}_2$  by an alkaline sodium hypobromite solution



The  $\text{CO}_2$  is bound by sodium hydroxide while the liberated nitrogen is determined volumetrically. From this the amount of urea present in the urine is calculated

*Reagents*

(1) saturated sodium chloride-sodium sulfate solution 100 Gm of sodium sulfate and 350 Gm of sodium chloride is heated to boiling with 1 liter of water allowed to cool and filtered.

(2) 30 per cent sodium hydroxide solution (prepared from NaOH purified with alcohol)

(3) bromine

(4) 10 per cent trichloroacetic acid or if no trichloroacetic acid is available, solutions

(5) 4.5 percent  $\text{ZnSO}_4$

(6) N/1 NaOH

*Apparatus*

The determination is best carried out in the Kowarski urometer. This consists of a U-shaped glass tube which may be divided into 3 compartments with the aid of 2 stopcocks (fig 31)

*Procedure*

Five milliliter of urine are pipeted into a wide test tube (or better a Hagedorn Jensen tube). Twice 5 ml of distilled water are added with the same pipet. This is followed by 5 ml. of trichloroacetic acid (4). The mixture is well shaken and filtered. If no trichloro

acetic acid is available the deproteinizing may be done as follows. To 5 ml. of urine are added 5 ml. of 4.5 per cent  $\text{ZnSO}_4$  (5), 1 ml. of N/1 NaOH and 9 ml. of distilled water. It is well shaken and filtered. Now the urometer is filled with the  $\text{NaCl}$   $\text{Na}_2\text{SO}_4$  solution, but first

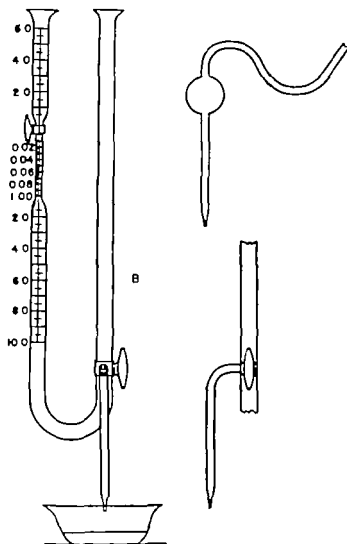


FIG. 31. KOWARSKI UROMETER

the upper stopcock is opened and the lower 3-way stopcock is adjusted so that the black dot points towards the rear. The solution is poured into the right arm until its level reaches above the upper stopcock. Now this stopcock is closed and the 3-way stopcock is adjusted so that the black dot points down. With the stopcock in

this position the left arm is connected with the draining tube and negative pressure is established in this part of the apparatus. With the accompanying pipet the liquid is removed from the upper part of the left arm and the clear filtrate from the test tube is introduced. The liquid level is noted now the upper stopcock is carefully turned and exactly 2.5 ml. of the filtrate is allowed to enter the lower part of the tube. The remainder of the filtrate is removed with the pipet, the tube is rinsed with distilled water which is again removed with the pipet, the washing is repeated. Now 10 ml. of 30 per cent NaOH is placed into a test tube. 20 drops of bromine are added from a dropping bottle and the mixture is poured back and forth several times from one tube into another. This sodium hypobromite solution is poured into the upper part of the left arm, the stopcock is opened and 5-9 ml. of hypobromite (according to the urea content) is allowed to enter the tube slowly. The liberated gas will push the NaCl  $\text{Na}_2\text{SO}_4$  solution out through the draining tube. After ten minutes when the gas development has ceased and the liberated nitrogen has cooled to room temperature, the 3 way stopcock is turned so that the black dot points again towards the rear. In this position both arms of the U are connected. Since the measurement of the gas must be carried out at atmospheric pressure the 3 way stopcock is slowly turned so that the black dot points upwards which causes the NaCl  $\text{Na}_2\text{SO}_4$  solution to drain from the right side. The liquid level is adjusted to the same height in both arms. The urea content is calculated as follows. The amount of liberated gas is noted exactly. In table 8 the figure is looked up which corresponds to the temperature and barometric pressure of the experiment. This figure multiplied by the figure found for the gas volume gives the amount of urea in mg. per cent.

#### *Example*

Three and five-tenths milliliters of gas are liberated at a temperature of 20° C. and a barometric pressure of 760 mm. Three and five-tenths multiplied by the figure found in the table equals amount of urea in milligrams per cent.

$$3.5 \times 392 = 1372 \text{ mg. per cent}$$

After the determination the contents of the left arm are removed by opening the upper stopcock and adjusting the 3 way stopcock.



U = urea concentration of urine (in mg per 100 ml)

B = urea concentration in blood (in mg per 100 ml)

V = urine volume per minute

Example for calculation of a "maximum urea clearance"

Urea concentration of urine (U) = 430 mg per cent

Urea concentration of blood (B) = 32.5 mg per cent

Urine volume per hour = 231 ml

Urine volume per minute (V) = 3.85 ml

$$C_{\text{m}} = \frac{U V}{B} = \frac{430 \times 3.85}{32.5} = 50.9 \text{ ml of blood cleared of urea per minute.}$$

$$\begin{aligned} \text{Percentage of average normal } C_{\text{m}} &= \frac{1.33 U V}{B} \\ &= 1.33 \times 50.9 = 67.69 \text{ per cent} \end{aligned}$$

Example for calculation of a 'Standard urea clearance'

Urea concentration of urine (U) = 1530 mg per cent

Urea concentration of blood (B) = 30 mg per cent

Urine volume per hour = 46 ml

Urine volume per minute (V) = 0.76 ml

$$\begin{aligned} C &= \frac{U \sqrt{V}}{B} = 51 \times \sqrt{0.76} = 51 \times 0.87 \\ &= 44.37 \text{ ml. of blood cleared of urea per minute.*} \end{aligned}$$

$$\begin{aligned} \text{Percentage of average normal } C_{\text{s}} &= \frac{1.85 U \sqrt{V}}{B} \\ &= 1.85 \times 44.37 = 82.08 \text{ per cent} \end{aligned}$$

According to van Slyke Page Hiller and Kirk (J Clin Invest. 14 901, 1935) results are somewhat more consistent if, in the formula given above one uses for "U" the urinary concentration of urea N

\* To facilitate the square root calculation see table 9. The square roots may be interpolated in the following way. For example the square root of 0.76 is to be determined. From the table it is seen that  $\sqrt{0.70} = 0.84$  and  $\sqrt{0.80} = 0.89$  i.e. between  $\sqrt{0.70}$  and  $\sqrt{0.80}$  the average value for  $\sqrt{0.1}$  is 0.05 or for  $\sqrt{0.01} = 0.005$ . If  $\sqrt{0.76}$  is to be calculated  $6 \times 0.005 = 0.03$  will have to be added to 0.84 = 0.87.

plus ammonia N instead of only urea N. The technical procedure is then also simplified since the process of removal of ammonia by permutite is eliminated. If this method is employed the following formulae are substituted for those given above:

$$\text{Percentage average normal } C_m = \frac{1.26 UV}{B}$$

$$\text{Percentage average normal } C = \frac{1.76 U\sqrt{V}}{B}$$

Normal values: 70-130 per cent of the average normal. The calculation of the urea clearance is simplified by the use of the line charts (fig. 32 a, b).

If the urine volume is less than 2 ml. the standard clearance is calculated and if the urine volume exceeds 2 ml. per minute the maximum clearance. When the line charts are used it is necessary to calculate by arithmetic only the value of the quotient  $\frac{U}{B}$  in terms of mg. per cent.  $V$  is estimated in ml. of urine per minute. A thread stretched across from the value  $\frac{U}{B}$  to  $V$  will cross the inner scale at a point indicating both the absolute clearance and the percentage of normal.

*Significance of the abnormal results.* The determination of the blood urea clearance is perhaps the most valuable and most accurate single method of detecting the presence and estimating the extent of renal function in efficiency. It cannot be employed to indicate the presence or nature of renal disease. In glomerulonephritis blood urea clearance may be assumed to indicate the proportion of glomerular tissue still functioning. In nephrosclerosis it may be assumed to indicate the relative adequacy of the renal circulation.

Significant alteration in blood urea N concentration usually does not occur before the urea clearance values fall to 50 per cent of the average normal.

Experience has shown that if a lowered clearance value in acute glomerulonephritis does not begin to rise steadily toward normal

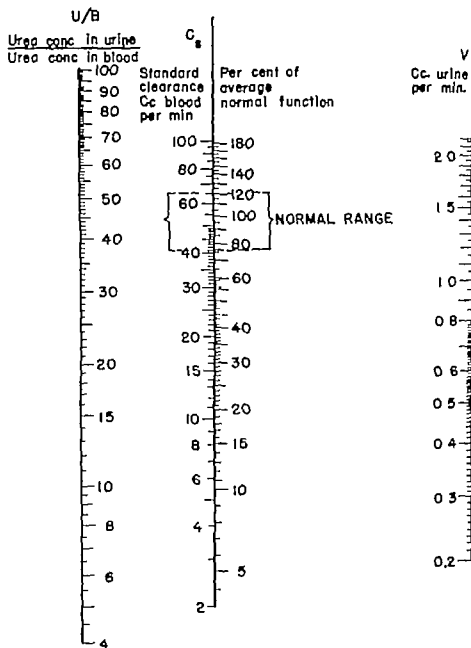


FIG 32a Line chart for the calculation of the urea clearance

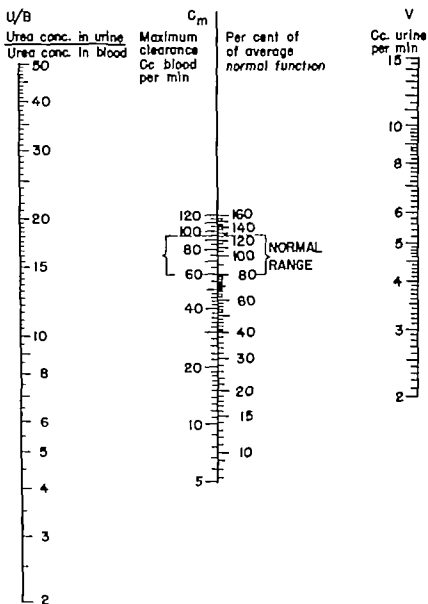


FIG 32b Line chart for the calculation of the urea clearance

within four months after the onset of the disease the process is probably progressing into the chronic active or terminal stages. Uremia almost invariably supervenes when the blood urea clearance falls to 5 per cent normal.

### DETERMINATION OF AMMONIA <sup>25-27</sup>

#### *Titrimetric Determination of Ammonia*

*Principle of the method* As suggested by J. K. Parnas and coll. the ammonia is liberated by sodium borate and absorbed in dilute

TABLE 9—*Table of Square Roots*

0.1 = 0.32	1.1 = 1.05
0.2 = 0.45	1.2 = 1.10
0.3 = 0.55	1.3 = 1.14
0.4 = 0.63	1.4 = 1.18
0.5 = 0.71	1.5 = 1.23
0.6 = 0.78	1.6 = 1.27
0.7 = 0.84	1.7 = 1.30
0.8 = 0.89	1.8 = 1.34
0.9 = 0.95	1.9 = 1.38
1.0 = 1.0	2.0 = 1.42

HCl. It is oxidized by the addition of a measured amount of NaOBr solution of known titer and the excess hypobromite is titrated with a known naphthyl red solution.

#### *Reaction equation*



The dye solution reacts like permanganate solution. The amount of dye solution used till the appearance of its own color is proportional to the excess bromine present. The naphthyl red method can only be used for amounts of ammonia ranging from 1-20  $\gamma$ .

#### *Reagents*

(1) Dilute NaOH is added to 20 ml. of a N/10 bromine-potassium bromide stock solution (see nonprotein nitrogen determination without distillation p. 144) until the brown color disappears, this is fol-

\* Mainly according to Torsten Theorell *Biochem. Zeitschr.* 248:246, 1933.

lowed by 50 ml of N/1 sodium carbonate solution and made up to 1 liter with distilled water. This solution can only be used a few hours after preparation, however it will keep for several days when stored in a cool and dark place.

(2) naphthyl red stock solution. In a 100 ml volumetric flask 100 mg of naphthyl red is dissolved in 5 ml of N/1 NaOH and made up to the mark with water.

(3) 5 per cent KBr solution.

(4) acid mixture. 60 ml of glacial acetic acid and 7.5 ml of concentrated phosphoric acid are mixed and made up to 100 ml with water.

(5) ammonium sulfate stock solution. 0.4716 Gm of pure ammonium sulfate is dissolved in 1 liter of water. Immediately before use this stock solution is diluted ten times with water. 1 ml of the dilute solution contains 10  $\gamma$  of ammonia nitrogen. This solution may also be prepared by a six fold dilution of the ammonia standard solution described under colorimetric semimicro method for the determination of nonprotein nitrogen (see p. 140). For example in a 15 ml volumetric flask 2.5 ml of this solution is made up to the mark with water.

(6) saturated sodium borate solution (pH = 9.2). This solution must be heated for thirty minutes before use.

(7) water, absolutely free from  $\text{NH}_3$  (purified with permanganate see p. 136).

### *Procedure*

Immediately after being drawn (otherwise ammonia from adenosine phosphoric acid will be liberated) 1-2 ml. of oxalated blood are transferred into the distilling flask of the below described apparatus (fig. 33). Four ml. of borate buffer (6) and a few drops of mineral oil are added and the ammonia is distilled for twelve minutes at a reduced pressure of 20-30 mm Hg. Into the receiver is placed 2 ml. of approximately N/200 HCl.

Apparatus and procedure (fig. 33)

The steam generator (1) is half filled with boiling water acidified with  $\text{H}_3\text{PO}_4$ . The 3-way stopcock (4) of the steam generator is adjusted to let the steam pass into the open air through the flask

(2) instead of into the distilling flask (5). The receiving flask (13) which has previously been washed with dilute sodium hypobromite solution and rinsed thoroughly with water is filled with approximately 2 ml of N/200 HCl. The sample is introduced into the distilling flask (5) through the funnel (7). Then 2 ml of mineral oil (to prevent foaming) and 4 ml. of saturated borate solution for alkalinizing

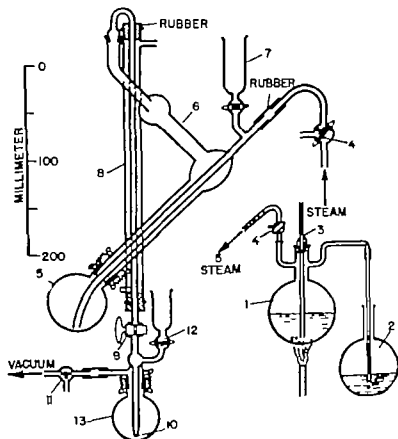


FIG. 33 Distilling apparatus for ammonia determination according to Thorsten Theorell

are added. Stopcocks (7) and (12) are closed, the 3-way stopcock (11) is so adjusted as to connect a water pump and mercury manometer with the apparatus. When a vacuum of 20-30 mm Hg has been reached the stopcock (4) is turned to permit the steam to reach the distilling flask (5) it is manipulated slowly and carefully and its position is adjusted so that the contents of the flask (5) begin to boil and the flask feels warm to the touch (40-60° C). A small in

dentation on the 3-way stopcock facilitates the adjustment. The stopcock (9) is adjusted so that the condensing droplets fall one by one into the receiver. In this manner moderate boiling in the receiver is achieved without loss by splashing. The stopcock (9) facilitates temperature regulation in the receiver as well as in the distilling flask independently from each other. If the stopcock has once been properly adjusted it will require only occasional resetting.

The distillation is usually completed after ten minutes, when 10-15 ml. of water have passed into the receiver. At this point the distillation is interrupted: the stopcock (4) is closed towards the apparatus, the vacuum stopcock (11) is opened towards the air, so that it is closed towards the receiver. Immediately after that the funnel stopcock (7) is opened slowly (watch for backing up of fluid), so that air may now enter the apparatus. The receiver is disconnected and the drops at the tip of the condenser tube are rinsed off with some water through the funnel (12). The titration is performed directly in the receiver flask or its contents are transferred into a small flask (it is better to have at hand several small exchangeable flasks all equipped with the same size ground glass joint to fit the apparatus). Stopcock (4) must not be greased, but should be moistened each time with water which can be introduced through the short airway of the stopcock. All other stopcocks and ground glass parts should be covered with a thin layer of yellow vaseline or mineral oil (all materials used must be tested for  $\text{NH}_3$ ). The steam generator (1) is filled from the flask (2) after removal of the plasma (negative pressure).

**Sources of error during distillation.** In this method of distillation an error may arise from the fact that the distilling flask is kept too cold so that not all the ammonia present will distill over within ten minutes. To avoid this the steam-stop cock (4) should be so adjusted as to keep the distilling flask slightly warmer than room temperature: this can be ascertained by touch. The steam generator may contain interfering materials. All rubber connections of the vacuum system (including those leading to the pump and manometer) must be kept as short as possible and the tubing should be boiled in vaseline in vacuo.

**Titration.** To the faintly acid distillate in the receiver flask (or after transfer to a 50 ml flask) are added exactly 2 ml of sodium

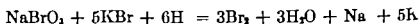


hypobromite solution drop by drop. After at least 30 seconds 1 ml of KBr solution and 1 ml of acid is added. The sample is again left for thirty seconds and is then titrated with shaking with the yellow naphthyl red solution until a faint pink color appears which remains stable for at least ten seconds. The change of the colorless solution to a faint pink (end of the titration, acid change of indicator) can be observed exactly to 0.02 ml with some experience.

Potassium bromide is added for two reasons:

(1) Bromine dissolves readily in aqueous solution of potassium-bromide forming a tribromide ion  $\text{Br}_2 + \text{Br}' = \text{Br}_3$ . The resultant solution has a much lower vapor pressure than the solution of bromine in pure water, consequently the loss by volatilization is considerably diminished.

(2) the  $\text{HBrO}_2$  resulting from a rearrangement of sodium hypobromite reacts with KBr to form bromine:



Since the naphthyl red requirement depends upon the bromine ion concentration and the pH, the acid as well as the KBr must be measured out very carefully. Exposure of the sample to direct sunlight should be avoided. Marbles or small watch glasses placed on top of the flasks protect from dust during the various manipulations. It is most important to titrate slowly. The 10 ml burette containing the naphthyl red solution should be equipped with a fine tip so that the draining time (with wide open stopcocks) for 10 ml is 60-70 seconds. After several analyses the titration flasks must be washed with alcohol and rinsed well with water. The distilling flasks must be washed with sodium hypobromite and water before each determination.

Sources of error in the titration: contamination material such as dirty flasks, dust, etc. will interfere with the titration.

The following condition may lead to errors: if excess ammonia is present it will not completely react with the hypobromite (rather oddly) on the contrary the bigger the excess of ammonia the more hypobromite is left without reacting. Consequently smaller amounts of ammonia than actually present in the sample will be found by the naphthyl red titration. If one is not certain whether the selected

amount of sodium hypobromite is sufficient for the  $\text{NH}_3$  present, a parallel experiment should be performed with one-half the amount of distillate or with an aliquot part of the distillate. This may be done as follows: the distillate is transferred quantitatively into a volumetric flask and made up to the mark with water. If the amount of  $\text{NH}_3$  is too large a more concentrated hypobromite solution is used. It is also possible to choose different concentrations of naphthyl red and of hypobromite. (With a N/3500 solution of naphthyl red prepared by diluting 12 ml of stock solution to 1 liter with water, exact color changes with one drop can be achieved as long as the total volume does not exceed 10 ml.)

If 0.4 ml. of N/500 sodium hypobromite solution is used and the titration is performed with N/3500 naphthyl red from a 2 ml. exact microburette values of 0.2–1  $\gamma$  can be detected very exactly, if the volume does not exceed 5 ml.)

### Calculation

(1) determination of the titer of the dye solution. 1 ml. of dilute standard solution is distilled. 2 ml. of NaOBr are added, and the amount of dye required is determined by titration. The amount of dye used up by 2 ml. of hypobromite solution is also determined. The difference (X) between the amount of dye required by the hypobromite solution and by the ammonium sulfate solution corresponds to 10  $\gamma$  of ammonia nitrogen. 1 ml. of dye solution corresponds to  $\frac{10}{X}$   $\gamma$  of ammonia nitrogen in the sample.

(2) calculation of the ammonia nitrogen in the blood sample. amount of dye used up by the blank minus amount of dye used up by the sample multiplied by  $\frac{10}{X}$  results in  $\gamma$  ammonia nitrogen.

### Example

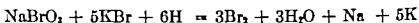
2 ml. hypobromite require	0.75 ml. of naphthyl red
1 ml. of ammonium sulfate sol. cor- resp. to 10 $\gamma$ N	4.18 ml. of naphthyl red
	<hr/>
10 $\gamma$ N require	5.57 ml. of naphthyl red
1 ml. of naphthyl red corresponds to	1.79 $\gamma$ nitrogen

hypobromite solution drop by drop. After at least 30 seconds, 1 ml. of KBr solution and 1 ml. of acid is added. The sample is again left for thirty seconds and is then titrated with shaking with the yellow naphthyl red solution until a faint pink color appears which remains stable for at least ten seconds. The change of the colorless solution to a faint pink (end of the titration, acid change of indicator) can be observed exactly to 0.02 ml. with some experience.

Potassium bromide is added for two reasons:

(1) Bromine dissolves readily in aqueous solution of potassium bromide forming a tribromide ion  $\text{Br}_2 + \text{Br}^- = \text{Br}_3^-$ . The resultant solution has a much lower vapor pressure than the solution of bromine in pure water, consequently the loss by volatilization is considerably diminished.

(2) the  $\text{HBrO}_2$  resulting from a rearrangement of sodium hypobromite reacts with KBr to form bromine:



Since the naphthyl red requirement depends upon the bromine ion concentration and the pH, the acid as well as the KBr must be measured out very carefully. Exposure of the sample to direct sunlight should be avoided. Marbles or small watch glasses placed on top of the flasks protect from dust during the various manipulations. It is most important to titrate slowly. The 10 ml. burette containing the naphthyl red solution should be equipped with a fine tip so that the draining time (with wide open stopcocks) for 10 ml. is 60-70 seconds. After several analyses the titration flasks must be washed with alcohol and rinsed well with water. The distilling flasks must be washed with sodium hypobromite and water before each determination.

Sources of error in the titration: contamination material such as dirty flasks, dust, etc. will interfere with the titration.

The following condition may lead to errors: if excess ammonia is present, it will not completely react with the hypobromite (rather oddly) on the contrary, the bigger the excess of ammonia, the more hypobromite is left without reacting. Consequently, smaller amounts of ammonia than actually present in the sample will be found by the naphthyl red titration. If one is not certain whether the selected

amount of sodium hypobromite is sufficient for the  $\text{NH}_3$  present, a parallel experiment should be performed with one-half the amount of distillate or with an aliquot part of the distillate. This may be done as follows: the distillate is transferred quantitatively into a volumetric flask and made up to the mark with water. If the amount of  $\text{NH}_3$  is too large a more concentrated hypobromite solution is used. It is also possible to choose different concentrations of naphthyl red and of hypobromite. (With a N/3500 solution of naphthyl red prepared by diluting 12 ml of stock solution to 1 liter with water exact color changes with one drop can be achieved as long as the total volume does not exceed 10 ml.)

If 0.4 ml of N/500 sodium hypobromite solution is used and the titration is performed with N/3500 naphthyl red from a 2 ml exact microburette, values of 0.2–1  $\gamma$  can be detected very exactly if the volume does not exceed 5 ml.)

### Calculation

(1) determination of the titer of the dye solution. 1 ml of dilute standard solution is distilled. 2 ml of NaOBr are added, and the amount of dye required is determined by titration. The amount of dye used up by 2 ml of hypobromite solution is also determined. The difference (X) between the amount of dye required by the hypobromite solution and by the ammonium sulfate solution corresponds to 10  $\gamma$  of ammonia nitrogen. 1 ml of dye solution corresponds to  $\frac{10}{X}$   $\gamma$  of ammonia nitrogen in the sample.

(2) calculation of the ammonia nitrogen in the blood sample. amount of dye used up by the blank minus amount of dye used up by the sample multiplied by  $\frac{10}{X}$  results in  $\gamma$  ammonia nitrogen.

### Example

2 ml hypobromite require	9.75 ml of naphthyl red
1 ml of ammonium sulfate sol cor resp to 10 $\gamma$ N	4.18 ml of naphthyl red
10 $\gamma$ N require	5.57 ml of naphthyl red
1 ml of naphthyl red corresponds to	1.70 $\gamma$ nitrogen

with 2 ml of hypobromite in the receiver the distillate of 2 ml of blood requires 8.70 ml. of naphthyl red, consequently the sample contains  $(9.75-8.70) \times 1.79 \gamma \text{ nitrogen} = 1.05 \times 1.79 = 1.87 \gamma \text{ N}$ . The ammonia nitrogen content of the blood is 0.0935 mg per cent.

### *Colorimetric Determination of Ammonia<sup>21-22</sup>*

*Principle of the method* Since the amount of ammonia present in blood is very small, its determination requires especially designed apparatus and very careful work. The ammonia is steam distilled in vacuo over a period of 3-4 minutes which has the same effect as if 150 liter of an indifferent gas had been sent through the sample being analyzed. Steam and ammonia are condensed together, absorbed in acid and the ammonia is determined colorimetrically with Nessler's reagent.

### *Reagents*

(1) saturated sodium borate solution, freed from ammonia by prolonged boiling. One ml of this solution will provide 2 ml. of blood with the pH suitable to prevent spontaneous formation of  $\text{NH}_3$  and equally suitable for the liberation of preformed  $\text{NH}_3$  (pH = 9.2)

(2) mineral oil

(3) N/10 HCl

(4) ammonia free sodium or lithium oxalate

(5) Nessler's reagent (see p. 140)

(6) ammonium sulfate standard solution containing 0.002 mg of nitrogen per ml = 200  $\gamma$  per cent. This solution is best prepared from the ammonium sulfate standard solution used in the colorimetric semi-micromethod for the determination of nonprotein nitrogen (solution 3, p. 140). In a 150 ml volumetric flask 5 ml of this standard solution is made up to the mark with water.

Apparatus see fig. 34

Steam is generated from a round bottom flask, containing dilute phosphoric acid and equipped with a 3-hole cork stopper. The steam passes through a pyrex stopcock (P) into the distilling flask (D). The excess steam is led below the water surface of a glass flask (W). Since rubber stoppers surrounded by hot water vapors

are omitted in this apparatus, no interference from sulfur compounds or ammonia from rubber stoppers can occur. Any rubber connections of the apparatus are to be kept very short or should be avoided entirely by fusing the glass parts together. A piece of silver mesh is inserted between (A) and (O) to retain any possibly present hydro-

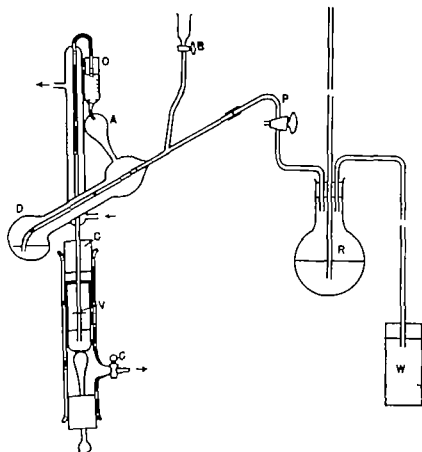


FIG. 34 Apparatus according to Parnas for the determination of ammonia in blood

gen sulfide. The receiver (V)\* is attached upon the glass support so that it may be placed higher or lower as needed. The condenser tube is made of silver. It is advantageous not to place the rubber stopper (C) holding the jacket of the receiver (V) directly upon the

The receiver flask should carry graduations so that the standard solution may be made up to the same volume as the sample.

silver tube, but to coat the silver tube with Khotinski cement when still warm and then slip the stopper over that layer of cement. Otherwise the silver corrodes from the sulfur of the rubber\* and the connection loosens. The diameter of the receiver should measure at least 16 mm and it should be made of heavy glass. It is highly recommended to maintain the indicated measurements. The most important factor in the arrangement made by Parnas is the use of controls to eliminate possible errors.

### *Procedure*

Each analysis is performed in several steps

(1) the reagents, i.e., borate and mineral oil, are distilled. The distillate (4 ml of water) is absorbed in the receiver in 2 drops of N/10 HCl and should not give more color with Nessler's reagent than 4 ml of distilled water. Otherwise the distillation must be repeated.

(2) now the blood is added and again 4-6 ml is distilled off. This represents the analysis of the sample.

(3) another 4 ml of water is distilled, this distillate must be either entirely free from ammonia or in cases of a very high ammonia content of the sample should contain not more than 0.1  $\gamma$ . If more ammonia is present, this distillate is added to the distillate of the analysis and the distillation repeated. This last distillate must be free from ammonia.

(4) a known amount of ammonia is added, and distilled, the added amount of ammonia must be recovered quantitatively.

Control (1) serves to test the reagents and the apparatus for traces of ammonia. Control (3) indicates the completion of the distillation and control (4) shows that at the pH of the borate even large amounts of ammonia can be recovered quantitatively.

### *Performance of the analysis*

The apparatus is connected as indicated in figure 34. The water pump is turned on the steam generator boils rapidly, and steam escapes to the outside through W. The stopcocks P, B, and C are closed the receiver contains 2 drops of N/10 HCl and the condenser tube reaches below the surface of the acid.

\* All rubber stoppers and tubings must be boiled in vaseline in vacuo

The funnel is filled with 2 ml of mineral oil and 1 ml of borate solution, stopcock C is opened and by careful opening of stopcock B oil and borate solution are allowed to enter the distilling flask. Then stopcock B is again closed. Now stopcock P is slowly opened to permit steam to enter the distilling flask. The exact position of the stopcock must be found out experimentally the temperature of the distilling flask serves as an indicator it should not rise above 30° C. Also the water from the receiver must not rise in the condenser tube. While adjusting the correct flow of steam the receiver is carefully lowered so that the end of the condenser tube just barely dips below the surface of the liquid. As soon as 4 ml have distilled over, the condenser tube is removed from the receiver to allow 1 ml of water to drop off freely. Stopcock P is closed then stopcock C and finally air is allowed to enter slowly through P. The receiver is removed from the apparatus, it is tested with Nessler's reagent for the presence of ammonia, and if the test is negative the actual analysis may be started.

Again 1 ml of mineral oil is poured into the funnel stopcock C is opened and exactly 1 ml. of oxalated blood is layered beneath the mineral oil. By careful, but rapid opening of the stopcock blood and mineral oil are introduced into the distilling flask. In this manner the sample is transferred quantitatively. Once the blood has entered the flask it is immediately hemolyzed and begins to foam because of gas liberation. Without further waiting the distillation is started by adjusting stopcock P so that the temperature of the blood mixture does not rise above 20° C. the temperature can be estimated by touching the flask. Rapid distillation will not cause any foaming of the alkalinized blood mixture foaming will only occur when the liquid becomes overheated and the steam is then suddenly turned off.

In this manner 6-8 ml. are distilled off, the stopcocks are closed as described above and now the control distillations 3 and 4 are carried out. If the ammonia content of the blood is less than 0.2 mg per cent 2 ml. of blood and 2 ml of borate solution are used, but not more than 6-8 ml are distilled over. Several blood samples may be distilled without emptying the distilling flask, but a control distillation (3) must be carried out each time. Each distillation will take 3-4 minutes.

One ml. of standard solution (6) is placed into a second receiving



tube and made up with water to the same volume as the sample. 0.25 ml. of Nessler's reagent is added to both tubes and the colors are compared in the colorimeter.

To empty and clean the distilling flask it is filled with water from B and the water is sucked off through the stopcock P. All residues from blood must be completely removed from the flask, whereas mineral oil residues may be left behind.

As an improvement according to Stojanowitsch<sup>21</sup> Parnas recommends to draw the blood from the vein immediately into a syringe filled with borate solution to prevent any spontaneous ammonia development.

### *Example*

2 ml. of blood used for analysis

1 ml. of standard (same volume as distillate) contains 2  $\gamma$

reading of standard 10

reading of sample 18

the ammonia content is calculated according to the formula

$$\frac{10 \times 2}{18} = \frac{20}{18} = 1.1 \gamma, \text{ in } 100 \text{ ml of blood } 1.1 \times 50$$

$$= 55 \text{ or } 0.055 \text{ mg per cent}$$

The normal value of ammonia is 0.05–0.1 mg per cent.

## DETERMINATION OF AMINO ACIDS<sup>22, 23</sup>

### *Determination of Free Amino Acids*

*Principle of the method* In the trichloroacetic acid filtrate of whole blood or serum the free amino acids are condensed with  $\beta$ -naphthoquinone sulfonic acid. The resulting yellow color, which in contrast to the yellow color of the sulfonic acid does not disappear upon addition of sodium thiosulfate in faintly acid solution, is compared in the Dubosque or Hellge colorimeter with a standard amino acid solution.

### *Reagents*

- (1) twenty per cent trichloroacetic acid
- (2) N/1 NaOH

(3) two-tenths per cent alcoholic phenolphthalein solution

(4) saturated borax solution

(5) sodium- $\beta$ -naphthoquinone sulfonate 0.05 Gm is dissolved in 20 ml of cold distilled water (to be prepared freshly for each determination) The reagent may only be used when its yellow color disappears upon the addition of thiosulfate

(6) amino acid standard solution

(a) stock solution 75 mg of glycocoll and 147 mg of glutamic acid (corresponding to 28 mg of nitrogen) are placed into a 200 ml volumetric flask, containing 40 ml of trichloroacetic acid (1) and is made up to the mark with water This stock solution (containing 0.007 mg N in 9 ml.) will keep over a long period of time.

(b) dilute working standard ( $\alpha$ ) for the Dubosque colorimeter 39 ml of 20 per cent trichloroacetic acid are added to 5 ml of stock solution and made up to 100 ml with water ( $\beta$ ) for the Hellige colorimeter To 10 ml of stock solution in a 100 ml volumetric flask are added 38 ml of 20 per cent trichloroacetic acid and made up to the mark with water (containing 0.014 N in 7 ml)

(7) 4 per cent sodium thiosulfate solution

(8) acid solution 4.5 parts of N/1 HCl 1 part of glacial acetic acid, 2.5 parts of a formalin solution containing 1.8 ml of 40 per cent formalin per 100 ml

### *Procedure*

( $\alpha$ ) for the Dubosque colorimeter One ml of water and 0.2 ml of blood or serum are accurately measured into a short test tube To this is added 0.8 ml of trichloroacetic acid with the aid of the pipet shown in figure 4 After centrifugation at high speed 1 ml of the supernatant is transferred to a dry test tube marked at a point indicating 6 ml Into a similar tube is placed 1 ml of dilute standard solution, to both tubes is now added 1 drop of phenolphthalein solution (3) and the solution is neutralized with N/1 NaOH until a faint red color is visible The red color is removed with a microdrop of a very dilute acetic or sulfuric acid and 0.4 ml of saturated borax solution is added Now all tubes receive 0.8 ml of naphthoquinone reagent and are placed in a dark place at room temperature for 16-24 hours After this time 0.5 ml of thiosulfate solution and 0.5 ml of acid mixture are added to each tube the tubes are made up to the mark with water and compared in the colorimeter

*Calculation*

$$\frac{\text{reading of the standard} \times \text{concentration of the standard}}{\text{reading of the sample}}$$

= amino acid nitrogen of the sample

This figure multiplied by 1000 (since 0.1 ml. of blood has been used for the determination) expresses the amino acid nitrogen in 100 ml. of analyzed sample.

When a series of analyses is to be performed, one standard must be run for every two unknown samples, as the color is not stable.

*Example*

reading of standard	20 mm.
reading of sample	18 ml
concentration of standard	0.007 mg N

$$\text{amino acid nitrogen content of sample} = \frac{20 \times 0.007}{18} = 0.0077$$

$$0.0077 \times 1000 = 7.7 \text{ mg per cent amino acid nitrogen}$$

for more rapid calculation see table 24 (appendix, p. 377)

( $\beta$ ) for the Hellige colorimeter. The procedure is identical with the procedure described for the Dubasque colorimeter, only standard solution  $\beta$  is used. Since the wedge is larger than the cup, a 15 ml. volumetric flask is used to make up the standard solution. 2.5 ml. of standard  $\beta$  is used and consequently 2.5 times the amount of all reagents, i.e. 1 ml. of borax solution, 2 ml. of naphthoquinone reagent, 1.25 ml. of thio-sulfate and 1.25 ml. of acid mixture are measured out and treated as above. When a series of determinations is to be performed one standard must be run for every two unknown samples.

*Calculation*

$$\frac{100 - y}{100} \times 14 = \text{mg per cent amino acid nitrogen}$$

$y$  = colorimeter reading

*Example*

$$y = 45 \quad \frac{100 - 45}{100} \times 14 = 7.7 \text{ mg per cent amino acid nitrogen}$$

*Determination of the Total Amino Acids (Polypeptides and Free Amino acids)\*†*

**Principle of the method** The polypeptides are hydrolyzed to free amino acids by refluxing with dilute sulfuric acid for several hours and the free amino acids are determined as described above. The difference between total amino acid nitrogen and free amino acid nitrogen represents the polypeptide nitrogen.

**Reagents**

(1) concentrated  $H_2SO_4$

(2) 10 per cent anhydrous sodium carbonate solution

All other reagents as described for the determination of free amino acids.

**Procedure**

Three milliliters of trichloroacetic acid filtrate (dilution 1:10) and 0.3 ml of concentrated  $H_2SO_4$  are refluxed for 7 hours. Flask and condenser should be connected by a ground glass joint (see fig. 35a). After that time the filtrate is neutralized with sodium carbonate (2) using phenolphthalein as indicator and air is sucked through the solution during 25 minutes with a water pump to eliminate volatile alkaline material (fig. 35b). To speed up this process the flasks are placed into a water bath of  $50^\circ C$ . Now the contents are again acidified with dilute sulfuric acid (to expel  $CO_2$ ) and after the flasks have been placed into the water bath of  $50^\circ C$  air is sucked through for 10 more minutes. The flasks are cooled to room temperature the contents are neutralized with sodium carbonate and transferred quantitatively into test tubes, marked at 12 ml. As described above 2 ml of this solution (representing 0.05 ml of serum) are analyzed for amino acid nitrogen.

**Calculation** ( $\alpha$ ) for the Dubosque colorimeter

$$\frac{\text{reading of standard} \times \text{concentration of standard} \times 2000}{\text{reading of sample}}$$

= amount of total amino acid nitrogen in blood in mg per cent

Mainly according to E. Becher and E. Hermann Muench med Wchnschr 78: 1060 and 2179, 1925

Total amino acid nitrogen minus free amino acid nitrogen equals amount of polypeptide-nitrogen  
 For rapid calculation see table 24, p 377

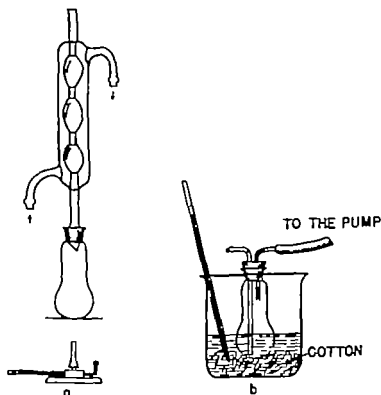


FIG. 35 Apparatus for the determination of polypeptide nitrogen a. Digestion flask with reflux-condenser b. Equipment for suction

### Example

Total amino acids

reading of standard	20 mm
reading of sample	24 mm
concentration of standard	0.007 mg

The result must be multiplied by 2, because 0.05 ml. of serum have been used for the analysis

$$\frac{20 \times 0.007 \times 1000 \times 2}{24} = \frac{70}{3}$$

= 11.7 mg. per cent total amino acid nitrogen.



(2) in eclampsia—during stages of kidney damage—amino acid values of between 6 and 12 mg per cent have been found

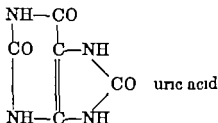
(3) in myeloid leukemia

In most cases of nephritis with increased nonprotein nitrogen the amino acid level is unchanged, but sometimes a rise in combined amino acids may be encountered under these conditions

## URIC ACID

### *Colorimetric Semimicro-determination*

Semi microdetermination in whole blood, serum and plasma according to Folin<sup>22</sup>



*Principle of the method* The deproteinized filtrate is heated with phosphotungstic acid in the presence of sodium cyanide. In the presence of uric acid a blue color will appear, originating from the reduction of the phospho-tungstic acid to a lower valence. The blue color is compared with the color produced under identical conditions by a uric acid standard solution of known concentrations.

### *Reagents*

- (1) 10 per cent sodium tungstate solution
- (2)  $\frac{2}{3}$  N  $\text{H}_2\text{SO}_4$  or 9.076 Gm of  $\text{KHSO}_4$  dissolved in 100 ml of water

(3) uric acid standard solution

(a) stock solution In a liter volumetric flask 1 Gm of uric acid (weighed on an analytic balance) and 0.6 Gm of lithium carbonate are dissolved in approximately 150 ml of water. (To accelerate the process the flask may be placed into warm water.) When the salts have completely dissolved, 20 ml of formalin (40 per cent) and approximately 500 ml of water are added. Now 25 ml of N/1  $\text{H}_2\text{SO}_4$  is added and made up to the mark with water. This solution should react acid towards neutral red and can be kept indefinitely when stored protected from daylight.

(b) working standard  $\alpha$ ) for the Dubosque colorimeter In a 250 ml volumetric flask 1 ml. of stock solution is made up to the mark with water Five ml. of this dilute standard solution contain 0.02 mg. of uric acid

( $\beta$ ) For the Hellige colorimeter in a 250 ml volumetric flask 2 ml. of stock solution are made up to the mark with water Five ml. of this dilute standard solution contain 0.04 mg. of uric acid

If a few drops of chloroform are added to these solutions, they will be stable for a short period of time when stored in a cool and dark place In order to be independent of the stability of the solution it is recommended to proceed as follows using a calibrated 0.1 or 0.2 ml pipet (see appendix p 362), 0.02 ml. of uric acid stock solution (3a) is transferred to the bottom of a dry test tube ( $\alpha$ ) when the Dubosque colorimeter is used, and 0.04 ml. of that solution when the Hellige colorimeter is available ( $\beta$ ) 4.98 ml. of water is added to tube ( $\alpha$ ) or 4.96 ml. to tube ( $\beta$ ) This is easily done if a 5 ml pipet is marked at the corresponding volumes

(4) sodium cyanide in a 200 ml volumetric flask 5.5 Gm. of sodium cyanide are dissolved in water with stirring and made up to the mark, 0.6 Gm. of calcium oxide is added and after 4-5 minutes shaking the solution is filtered. Before use, 9 parts of this solution are mixed with 1 part of 10 per cent  $\text{Na}_2\text{HPO}_4$  solution and filtered

(5) uric acid reagent

(a) in a 500 ml volumetric flask 100 Gm. of sodium tungstate are completely dissolved in 200 ml. of water with outside cooling and stirring 20 ml. of 85 per cent phosphoric acid is added Then a slow stream of hydrogen sulfide is blown through the mixture for 20 minutes At the end of the 3rd or 4th minute another 10 ml. of 85 per cent phosphoric acid are added Now the solution is filtered through cotton the first 40 ml. being refiltered through the same filter The filtrate is transferred to a 1 liter separatory funnel 300 ml. of pure ethanol is added and the mixture is shaken for several minutes. After the layers have separated, the upper layer is discarded and the lower layer is transferred to a tared 500 ml. florence flask and made up to 300 Gm. with water Now the flask is heated over a flame to remove the hydrogen sulfide Twenty ml. of concentrated phosphoric acid are added and the solution is refluxed for 1 hour several drops of bromine are added and the excess bromine is removed by heating



A molybdate-free sodium tungstate may also be prepared in solid form as follows

A solution of 250 Gm of sodium tungstate in 500 ml of water is treated with 5 N HCl until neutral to litmus paper. The solution is saturated with hydrogen sulfide and allowed to stand for twenty-four hours. It is then treated with 400 ml of absolute alcohol, added gradually with constant shaking. The mixture, after standing for a further 24 hours, is filtered, and the precipitate is washed with 50 per cent alcohol and dissolved in 375 ml of water. Five-tenths ml of bromine is added and the mixture boiled gently until the excess bromine is expelled. Sodium hydroxide solution (40 Gm per 100 ml) is now added to the hot solution until the latter is alkaline to phenolphthalein. The cooled solution filtered if necessary, is treated with 200 ml of absolute alcohol and allowed to stand for twenty-four hours. The white crystals are filtered off and dried in a desiccator.

If pure molybdate-free sodium tungstate c.p.\* is available, the reagent may simply be prepared as follows: a 100 Gm pure sodium tungstate is dissolved in 750 ml of water and with outside cooling and shaking 50 ml of 85 per cent phosphoric acid is added gradually. The solution is heated for one hour, the evaporating water being replaced during that time. Then several drops of bromine are added and the solution is again heated to destroy the excess bromine.

(b) to 12 Gm of lithium carbonate in a 500 ml beaker 25 ml of concentrated phosphoric acid and 150 ml of water is added. The lithium phosphate solution is heated to remove the liberated  $\text{CO}_2$  which would interfere with the analysis. Care must be taken not to leave any particles of lithium carbonate adhering to the sides of the beaker.

After cooling solutions (a) and (b) are pooled and made up to 1 liter with water. This solution will keep for a long period of time when stored in a dark bottle and in a cool place.

The reagent must be tested by a simple titration. The maximum color intensity is reached when 1 ml of reagent is mixed with up to 1.5 ml of 0.1 N/1 NaOH (avoid  $\text{Na}_2\text{CO}_3$ ) with phenolphthalein as indicator. If the reagent is too acid, NaOH must be added to the calculations if it is too alkaline  $\text{H}_3\text{PO}_4$  is a

Reagents 3, 4 and 5 must be in the ice

\* Test for molybdenum is as follows

*Procedure*(1) *Deproteinizing* (Folin Wu)

In a 100 ml. volumetric flask one part (2-3 ml.) of oxalated blood plasma or serum is diluted with 7 parts of distilled water and 1 part of 10 per cent sodium tungstate solution is added. One part of  $\frac{1}{2}$  N  $\text{H}_2\text{SO}_4$ \* is now added with shaking from a burette or pipet, the mixture is shaken until a metallic sound can be heard and after 2-3 minutes it is filtered through a folded filter. ( $\text{N}/12 \text{H}_2\text{SO}_4$  may also be used, see nonprotein nitrogen determination p. 140)

(2) *Estimation*

(a) with the Dubosque colorimeter. Five ml. of protein free filtrate corresponding to 0.5 ml. of undiluted starting material are placed into a test tube. A similar tube is filled with 5 ml. of dilute uric acid standard solution (3b a). Five ml. of cyanide-phosphate solution (using a special pipet fig. 36) and 1 ml. of uric acid reagent are added to each of both tubes. The contents are well mixed and allowed to stand at room temperature for four minutes. Then the tubes are placed into a boiling water bath for two minutes. The tubes are cooled rapidly with running water and the colors are compared in the Dubosque colorimeter.

*Calculation*

$$\frac{\text{reading of standard} \times \text{concentration of standard} \times 200}{\text{reading of sample}} = \text{uric acid content in mg. per cent}$$

*Example*

reading of standard	20 mm.
reading of sample	15 mm.
concentr. of standard	0.02 mg.

$$\frac{20 \times 0.02 \times 2 \times 100}{15} = 5.3 \text{ mg. per cent uric acid.}$$

(b) with the Hellge colorimeter. Five milliliters of protein-free filtrate are placed into a test tube. A second test tube (diameter approximately 22 mm.) is filled with 10 ml. of dilute uric acid standard solution (3b b). The rest of the procedure is identical with the

Instead of  $\frac{1}{2}$  N  $\text{H}_2\text{SO}_4$  a solution of 9.076 Gm. per cent  $\text{KH}_2\text{SO}_4$  may be used.

procedure described for the Dubosque colorimeter, only the standard tube is filled with double the amount of all reagents.

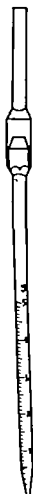


FIG. 33 Gift pipet. The bead in the wide part of the pipet prevents swelling of the liquid.

### Calculation

$$\frac{(100 - y) \times 8}{100} = \text{mg per cent uric acid}$$

$y$  = colorimeter reading

### Example

$$y = 34 \quad \frac{(100 - 34) \times 8}{100} = 0.66 \times 8 = 5.28 \text{ mg. per cent uric acid}$$

This value multiplied by 0.333 represents the uric acid nitrogen in mg per cent

*Micro-method in Nonhemolyzed Blood<sup>28</sup>*

*Reagents*

(1) sodium tungstate In a 500 ml volumetric flask 10 Gm of sodium sulfate is dissolved in water Twelve milliliters of 10 per cent sodium tungstate solution is added and all is made up to the mark with water

(2) 12 ml of  $\frac{1}{3}$  N  $\text{H}_2\text{SO}_4$  is made up to 100 ml with water

(3) uric acid standard solution ( $\alpha$ ) for the Dubosque colorimeter 1 ml of concentrated stock solution (see semimicromethod) is made up with water to 500 ml Four ml of this dilute solution contain 0.008 mg of uric acid Instead of this solution the dilute standard solution (3b,  $\alpha$ ) of the semi-micromethod may be employed, but it must be diluted 1:1 with water before use

( $\beta$ ) for the Hellige colorimeter 2 ml of stock solution (see semimicromethod) is diluted to 500 ml with water Four ml of this dilute solution contain 0.016 mg of uric acid Instead of this solution the dilute standard solution (3b,  $\beta$ ) of the semi-micromethod may be employed but it must be diluted 1:1 with water before use

If a few drops of chloroform are added to these solutions they will keep for some time when stored in a cool and dark place

(4) and (5) sodium cyanide solution and uric acid reagent are the same as described for the semi micromethod

*Procedure*

(1) deproteinizing

With an exactly calibrated capillary pipet 0.2 ml of blood is drawn from the fingertip The outside of the pipet is wiped off carefully and the blood is delivered into a test tube (shape and size see fig 37a) filled with 4 ml of sodium tungstate solution The pipet is rinsed 2-3 times by sucking up and blowing out of the tungstate solution The tube is allowed to stand at room temperature for at least fifteen minutes then 1 ml of  $\text{H}_2\text{SO}_4$  (2) is added drop by drop and with shaking and the tube is centrifuged at high speed within fifteen minutes.

(2) *estimation* ( $\alpha$ ) in the Dubosque colorimeter 4 ml of clear supernatant are carefully transferred to a dry test tube. Four ml of standard solution 3 $\alpha$  are measured into a similar tube. Five ml of sodium cyanide-phosphate solution and 1 ml of uric acid reagent are added to both tubes, the tubes are allowed to stand at room temperature for four minutes, whereupon they are heated for two minutes in a boiling water bath. They are rapidly cooled and the colors are compared in the Dubosque colorimeter.

### Calculation

$$\frac{\text{reading of standard} \times 5}{\text{reading of sample}} = \text{mg per cent uric acid}$$

the factor 5 is obtained as follows 0.2 ml of blood have been diluted to 5 ml of this dilution 4 ml corresponding to 0.16 ml of blood, have been used for the analysis. To find the value for 100 ml, the quotient  $\frac{\text{reading of standard}}{\text{reading of sample}}$  must be multiplied by  $\frac{0.008 \times 100000}{16}$   
= 5

See also table 24, p 377

### Example

reading of standard	20 mm.
reading of sample	18 mm
concentration of standard	0.008 mg

$$\frac{20 \times 5}{18} = 5.5 \text{ mg per cent uric acid}$$

( $\beta$ ) in the Hellige colorimeter 4 ml of supernatant are carefully transferred into a test tube. Into a second larger tube (diameter approximately 22 mm) 8 ml of dilute standard solution is measured (3 $\beta$ ) The rest of the procedure is identical with the procedure described for the Dubosque colorimeter except that the standard tube is filled with double the amount of all reagents

### Calculation

$$\frac{(100 - y) \times 10}{100} = \text{mg per cent uric acid.}$$

$y$  = colorimeter reading

*Example*

$$y = 45, \frac{(100 - 45) \times 10}{100} = 5.5 \text{ mg per cent uric acid}$$

*Micro-determination of Uric Acid in Serum, Plasma and Hemolyzed Whole Blood**Reagents*

(1) in a 100 ml volumetric flask 20 ml of 10 per cent sodium tungstate solution are made up to the mark with water

(2) in a 100 ml volumetric flask 25 ml. of  $\frac{1}{4}$  N  $\text{H}_2\text{SO}_4$  are made up to the mark with water

For solutions (3) (4), and (5) see colorimetric semi-micro determination of uric acid p 192

*Procedure*

(1) Deproteinizing Into a short test tube containing 0.8 ml. of sodium tungstate solution (1) 0.2 ml of whole blood serum, or plasma is pipetted, and the pipet is rinsed by repeated sucking up and blowing out of the mixture. One ml of  $\text{H}_2\text{SO}_4$  is added, mixed carefully with a glass rod and centrifuged

(2) Determination Into a large test tube are placed 1 ml of supernatant 1 ml of freshly prepared sodium cyanide-phosphate solution (4) and 0.2 ml of uric acid reagent (5). Simultaneously the following reagents are placed into another test tube 0.04 ml of concentrated stock solution (3), 4.96 ml of water, 5 ml of sodium cyanide-phosphate solution (4) and 1 ml of uric acid reagent (5). The contents of both tubes are mixed and the tubes are allowed to stand at room temperature for four minutes. Then they are immersed in a boiling water bath for two minutes and cooled rapidly with running water. The colors are compared in the Dubosque or Hellige colorimeter

In this method 0.1 ml of sample has been diluted to 2.2 ml. by the addition of all reagents. In this concentration of uric acid the color is intense and can be read easily

*Calculation*

(a) for the Dubosque colorimeter

$$\frac{\text{reading of standard} \times 8}{\text{reading of unknown sample}} = \text{mg per cent uric acid}$$

*Example*

reading of standard	20 mm
reading of sample	30 mm.
concentr of standard	0.008 mg

$$\frac{20 \times 8}{30} = 5.3 \text{ mg per cent uric acid}$$

(β) for the Hellige colorimeter

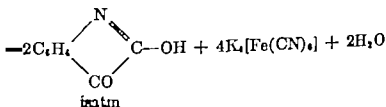
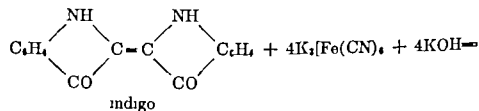
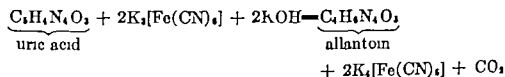
$$\frac{(100 - y) \times 8}{100} = \text{mg per cent uric acid, } y = \text{colorimeter reading}$$

*Example*

$$y = 35, \frac{(100 - 35) \times 8}{100} = 5.2 \text{ mg per cent uric acid}$$

*Titrimetric Determination of Uric Acid<sup>27</sup>*

*Principle of the method* One mol of uric acid is oxidized to allantoin by 2 moles of potassium ferricyanide in the cold. The excess potassium ferricyanide is titrated with sodium indigo sulfonate, in this reaction the ferricyanide oxidizes the blue dye solution to isatin which is colorless in this dilution.

*Reaction equation*

*Reagents*

- (1) 10 per cent sodium tungstate solution
- (2)  $\frac{1}{2}$  N  $\text{H}_2\text{SO}_4$  or 9.076 Gm per cent of  $\text{KHSO}_4$
- (3) potassium ferric cyanide solution 0.2348 Gm of potassium ferric cyanide is dissolved in 1000 ml of water Two moles of ferric cyanide correspond to 1 mol of uric acid consequently

$$\text{mg uric acid} = \frac{0.2348 \times 168}{2 \times 329.10} = 60$$

168 = molecular weight of uric acid 329.10 = molecular weight of K ferricyanide. One ml of this solution corresponds to 0.03 mg of uric acid

This solution will keep indefinitely when stored in a dark bottle and in a cool place

- (4) cold saturated sodium carbonate solution, approximately 20 per cent.

- (5) sodium indigo sulfonate 0.22 Gm of sodium indigo sulfonate is dissolved in 100 ml of a 3 per cent sodium fluoride solution and the mixture is made up to the mark with water in a 1000 ml volumetric flask. Before use the indigo solution is diluted 3-fold with water

*Procedure*

According to Folin Wu (see p. 190) 2-3 ml of serum are deproteinized To 5 ml of the filtrate (corresponding to 0.5 ml of serum) is added in a wide colorless test tube 1 ml of sodium carbonate and 1 ml of potassium ferric cyanide solution After 1-3 minutes the dilute blue indigo solution is added from a microburette with exact subdivisions, against a white background The tube is observed in reflected and transmitted light With some practice it is possible to determine the color change exactly with one single drop The titration is over when the blue color remains stable for thirty seconds. For each series of analyses the titer of the dye solution must be determined to eliminate errors caused by difference in lighting and by decomposition of the indigo solution To this purpose 1 ml of ferric cyanide in a wide test tube is diluted with 5 ml of water and 1 ml of sodium carbonate solution and this mixture is titrated with the indigo solution until a lasting blue color is noted Diffuse daylight offers the best conditions for the titration The indigo solution



should be diluted so that 1 ml of ferricyanide requires approximately 3 ml of dye solution

### Example

1 ml of ferric cyanide solution (corresponding to 0.06 mg of uric acid requires (A)	2.85 ml of indigo solution
5 ml of Folin Wu filtrate (corre- sponding to 0.5 ml of serum re- quire (B)	2.15 ml of indigo solution
	<hr/>
difference (corresponding to the use of oxydised uric acid	0.70 ml of indigo solution

The uric acid in 0.5 ml of serum is calculated according to the following formula

$$\frac{A}{A - B} = \frac{0.06}{X}$$

$$\frac{2.85}{0.70} = \frac{0.06}{X} \quad X = \frac{0.7 \times 0.06}{2.85} = 0.0147 \text{ mg}$$

Thus 0.5 ml of analysed serum contains 0.0147 mg of uric acid. This figure multiplied by 200 gives 2.94 mg of uric acid in 100 ml of serum. If this figure is multiplied by 0.333, the uric acid nitrogen is obtained. The following general formula represents the calculation

$$\text{mg per cent uric acid} = \frac{0.06 \times 100}{M} \times \frac{A - B}{A}$$

M = number of ml serum used for sample

A = indigo used up for titration of potassium ferric cyanide

B = indigo used up for uric acid determination in the blood filtrate

The entire determination requires only a few minutes.

The values found for uric acid are somewhat higher when this method is used than when the Folin method is employed. This method is recommended where work with the very toxic sodium cyanide should be avoided for safety reasons.

The normal uric acid content is 2-4 mg per cent. Uric acid

represents the end product of endogenous and exogenous nucleoprotein metabolism. Adenosine phosphoric acid of muscle is also a source of uric acid. Upon ingestion of a diet rich in nucleoproteins or meat (sweetbreads, brains) the uric acid level rises (exogenous component). When uric acid is to be determined, the patient should be on a nucleoprotein free diet (vegetable) for three days previous to the test.

A high uric acid level in blood may be caused by

- (1) decreased uric acid excretion
- (2) primary change of uric acid metabolism (gout)
- (3) increased endogenous nucleoprotein-decomposition
- (4) increased intake of nucleoproteins in individuals with impaired uric acid excretion or metabolism

In acute or subacute nephritis an increase in uric acid (4-10 mg per cent) may be encountered, often even before a rise in the other nonprotein nitrogen fractions is noted, this is due to diminished excretion through the kidneys. Amounts above 15 mg per cent are not rare. In cases of gout a uric acid content of 6-10 mg per cent can be found without a simultaneous increase in urea and creatinine. This holds true for uncomplicated cases and before and during the acute attack. As nephritis is a common complication of chronic gout a rise in the other nonprotein nitrogen fractions may accompany the uric acid retention. High uric acid values are observed in leukemias especially in myeloid leukemia, caused by a rise in endogenous nucleoprotein metabolism. The uric acid level is high in pernicious anemia during periods of remission parallel to the increase of reticulocytes. On the other hand when the disease is more severe, the uric acid level is lowered. The uric acid level is high in all cases of oliguria and anuria, in cases where the urethra is occluded by stones, in reflectory anuria, and in all diseases which are accompanied by destruction of kidney tissue, such as tuberculosis pyonephrosis, hydronephrosis, cystic kidney etc., also in arthritis deformans without true gout.

An increase in uric acid is frequently found in chronic lead poisoning. In eclampsia a rise in uric acid occurs accompanied by a slight rise in the other nonprotein nitrogen fractions. Finally increased uric acid values are found with all forms of cardiac decompensation and in constipation. In these cases a severe chronic nephritis is frequently the cause of the high uric acid level.

DETERMINATION OF CREATINE AND CREATININE<sup>31</sup>*Determination of Preformed Creatinine*

**Principle of the method** An alkaline picrate solution is added to the protein free filtrate and the resulting orange color is compared with a creatinine standard solution in a colorimeter

*Reagents*

(1) 10 per cent sodium tungstate solution

(2)  $\frac{1}{2}$  N  $\text{H}_2\text{SO}_4$  or a solution of 9.076 Gm per cent of  $\text{KHSO}_4$

(3) picric acid reagent (a) saturated solution of pure picric acid. Commercial picric acid must be purified according to the method of Benedict.<sup>32</sup> Commercial picric acid is dried in a vacuum-desiccator at 80° C or 90° C. 100 Gm of dry acid is dissolved in glacial acetic acid with warming and is then heated to boiling on a hot-plate. The hot solution is filtered through a preheated funnel with folded filter into a beaker. The beaker is covered with a watch glass and allowed to stand at room temperature over night. If after this time the picric acid has not crystallized out it is stirred vigorously or it is seeded with a few crystals of picric acid. Two hours after the beginning of crystallization it is filtered off by suction through hardened filter paper and washed with 35 ml of glacial acetic acid in small portions. The crystals are freed from glacial acetic acid by suction and dried in vacuo. Yield approximately 60 Gm of pure picric acid.

(b) 10 per cent sodium hydroxide

Immediately before use 25 ml of reagent (a) are mixed with 5 ml of reagent (b)

(4) creatinine standard solution (may be used for creatinine and creatine determination)

(a) stock solution in a 100 ml volumetric flask 0.1 Gm of pure creatinine is dissolved in 10 ml of N/1 HCl and made up to the mark with water

(b) working standard solution (a) for the Dubosque colorimeter In a 1 liter volumetric flask 6 ml of stock solution are mixed with 10 ml of N/1 HCl and made up to the mark with water. One ml of this solution contains 0.006 mg of creatinine

(b) for the Hellige colorimeter In a 1 liter volumetric flask 12 ml of stock solution is mixed with 10 ml of N/1 HCl and made up to the mark with water. One ml. of this solution contains 0.012

mg of creatinine Four to five drops of toluene are added to this solution as preservative

### Procedure

A test tube is filled with 5 ml of Folin Wu blood filtrate (see uric acid determination p 195) In a second tube 2.5 ml of creatinine standard solution (6) are diluted with water to 5 ml Two-tenths milliliters of freshly prepared alkaline picrate solution are now added to both tubes. If the sample is expected to contain much creatinine, 5 ml of standard solution are used The maximal color intensity is reached after 9-10 minutes and the colors are compared in the Dubosque colorimeter with the standard set at 20 or in the Hellige colorimeter The reading should be performed latest fifteen minutes after the picric acid reagent has been added

*Calculation* (a) for the Dubosque colorimeter

$$\frac{\text{Reading of standard} \times \text{concentration of standard}}{\text{reading of unknown sample}}$$

= creatinine content of sample in mg

If the above figure is multiplied by 200 (5 ml of filtrate corresponding to 0.5 ml of serum analyzed) the creatinine content of the sample is obtained expressed in mg per cent.

### Example

reading of standard	20 mm.
reading of sample	25 mm
creatinine content of standard	0.015 mg

$$\frac{20 \times 0.015 \times 200}{25} = 2.4 \text{ mg per cent creatinine}$$

*Calculation* (b) for the Hellige colorimeter

If a ready made wedge by the Hellige firm is employed, the standard solution is superfluous otherwise twice the indicated amount of standard solution 46β is used as well as twice the amount of alkaline picrate solution.

$$\frac{(100 - y) \times 6}{100} = \text{mg per cent creatinine}$$

y = colorimeter reading

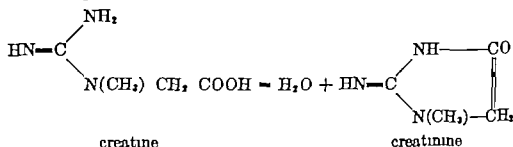
*Example*

$$y = 60, \quad \frac{(100 - 60) \times 6}{100} = 2.4 \text{ mg per cent creatinine.}$$

If the above figure is multiplied by 0.37, the creatinine-nitrogen is obtained. Better reading is obtained using the photoelectric colorimeter, see p. 395.

*Total Creatinine (Creatinine and Creatine)*

*Principle of the method* In the protein free blood filtrate creatine is transformed to creatinine by heating with dilute HCl in the autoclave (Arta pot, 3rd knob, p. 302, fig. 52).

*Reaction equation**Reagents*

(1) N/1 HCl, and all reagents described for creatinine determination.

*Procedure*

Two and five-tenths milliliters of Folin Wu filtrate (see p. 195) are measured into a test tube marked at 12.5 ml. After the addition of 0.5 ml of N/1 HCl the tube is covered with a small crystallizing dish and heated either in the Arta pot (3rd knob, fig. 52) or in the autoclave for 20 minutes at 130° C. After cooling 2.5 ml of alkaline picric acid solution are added. For the preparation of the standard solution 5 ml of standard  $\alpha$  or  $\beta$ , corresponding to 0.03 mg. of creatinine or 0.03 mg. of creatine are mixed with 0.5 ml of N/1 HCl and 2.5 ml of picric acid reagent. After standing for 5-10 minutes both tubes are filled up to the mark with water.

*Calculations* ( $\alpha$ ) for the Dubosque colorimeter

$$\frac{\text{reading of standard} \times 0.03}{\text{reading of sample}} = \text{mg creatinine}$$

The above value multiplied by 400 expresses the creatinine in mg per cent (2.5 ml of filtrate corresponding to 0.25 ml of original sample have been used for the analysis)

( $\beta$ ) for the Hellige colorimeter

$$\frac{(100 - y) \times 24}{100} = \text{mg per cent total creatinine}$$

$y$  = colorimeter reading

The difference between total creatinine and preformed creatinine gives the creatine content of the sample.

### *Qualitative Rapid Method for the Determination of Creatinine in Blood<sup>25</sup>*

*Principle of the method* The amount of creatinine in blood is estimated with the Jaffe color reaction

#### *Reagents*

- (1) saturated picric acid solution
- (2) 10 per cent sodium hydroxide

#### *Procedure*

Into a test tube containing 8 ml of saturated aqueous picric acid solution 2 ml of blood are delivered with a syringe immediately after having been drawn from the vein. The contents are mixed and warmed till a color change from yellow red to brown red is observed. Then it is filtered. The filtrate, which at times may be cloudy, is cooled with running water. For each ml of filtrate 1 drop of 10 per cent NaOH is added (it is not necessary to maintain quantitative proportions. When analysis is careful, less blood is needed)

#### *Estimation*

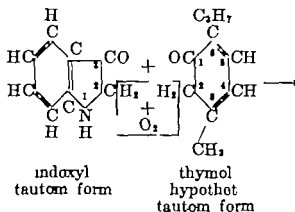
If the creatinine content is normal a color change of the picric acid from light yellow to dark yellow will occur. In these cases the creatinine content is too small to produce a distinct red color. When

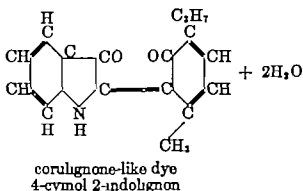
the creatinine concentration is slightly increased, a red tint will develop within five minutes. When the amount of creatinine present in the sample is above 10 mg per cent (corresponding to a nonprotein nitrogen content of more than 100 mg per cent) a deep red color develops within this time which covers up the original yellow color of the picric acid. With a certain experience, the intermediary shades of color between this picture and the just about noticeable red hue indicating a very small increase in creatinine—possibly even without elevated nonprotein nitrogen—can be recognized.

The normal serum content of creatinine is 1-2 mg per cent, that of creatine is 3-4 mg per cent. A rise is usually found in cases where the uric acid is also increased. Since creatinine is excreted more easily than uric acid, its increase in blood is supposed to be a sign of impaired kidney function. A high creatinine level during acute nephritis is by no means a very dangerous sign, whereas high values in chronic nephritis with uremia suggest an unfavorable prognosis. An increase in creatinine is also encountered in all cases of urinary retention, in cardiac decompensation and in intestinal obstruction.

### DETERMINATION OF INDICAN<sup>11</sup>

The indican-determination in blood is usually performed according to the classical method of A. Jolles<sup>2,3</sup> the method is based on the fact that indoxyl-sulfuric acid is condensed with thymol, then oxidized with Obermayer's reagent (ferrichloride and concentrated HCl). The indican is converted to a new indoxyl derivative whose chemical constitution Jolles showed to be 4-cymol 2 indolignone.





Jolles also found that indolignone dyes are obtained when thymol is replaced by its derivatives or by other aromatic phenols, provided the hydrogen atom in ortho-position to the OH-group is reactive and not substituted by other radicals.

*Principle of the method* Blood or serum is deproteinized with tri chloroacetic acid. A known amount of thymol or brominated thymol is added to the protein free filtrate. (For the standard tubes Br thymol is used as it has been shown that the thymol indican compound decomposes after a much shorter time than the corresponding Br thymol compound. Routine determinations may be carried out using thymol as Jolles has demonstrated, that the sensitivity of the reaction and the quality of the color are the same as when Br thymol is used). When indican is present a purple dye develops with Obermayer's reagent. At the same time trichloroacetic acid and the alcohol of the (Br) thymol in the presence of concentrated HCl form a compound which appears as oily drops in the reaction mixture and which absorbs quantitatively any dye formed (the isolation of the dye by extraction with chloroform is avoided in this manner). The oil drops will also appear in the absence of thymol, i.e. they are formed by trichloroacetic acid-ethyl ester. The reaction times being equal the size of the oil drop is directly proportional to the concentration of trichloroacetic acid, alcohol and HCl. The speed of the ester formation is most rapid in the first twelve hours after that time it is very small, but the reaction terminates only after approximately two weeks. The oil drop should have a size of 0.01 ml.

In order to obtain uniformly sized drops when new reagents are used—which is absolutely necessary for accurate results—the amount of thymol added is changed. The droplet size is determined in a



test tube with ground glass top (fig 37c). The bottom of the tube is drawn out to a capillary, which is graduated in 0.01 ml over a range of 0.1 ml.

### Reagents

(1) 40 per cent trichloroacetic acid (exactly). For the preparation of this solution Merck's crystallized trichloroacetic acid is used, and it is taken directly from a newly opened flask.

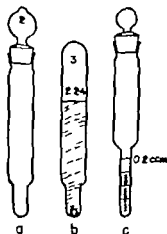


FIG. 37 Test tubes for indican determination

(2) (a) Thymol bromide. A solution of 2.2 ml of bromine in 25 ml of ether is added drop by drop and with cooling to a solution of 6 Gm of thymol in 25 ml of ether. The reaction product is freed from HBr by shaking with water in a separatory funnel, the ether layer is separated and dried with anhydrous  $\text{Na}_2\text{SO}_4$  over night. After filtration the ether is evaporated at low temperature (vacuum may also be used). The oily residue (thymol bromide) is dissolved in 100 ml of 95 per cent ethanol to which 1 ml of formic acid has been added (specific gravity 1.2). According to Plancher,<sup>41</sup> an excess of bromine is used in this method to assure complete conversion of thymol to thymol bromide. A small amount of dibromide is formed together with the monobromide. In this way of preparation the second bromine atom will occupy the ortho-position to the OH group, since thymol bromide which has been prepared under identical conditions, but with twice the amount of bromine cannot be condensed with indican. Formic acid is added to combine with free



and followed by 3 ml of Obermayer solution (3). The contents are again mixed and the tubes are corked. After approximately twelve hours they are sealed and left for two weeks in a cool dark place. After that time they are again mixed well and centrifuged. The series is now ready for the estimation, and may be kept for some time when stored in a cool and dark place.

In order to prepare test tubes producing droplets of the same size, the amount of thymol bromine must be increased in the following way: a number of tubes (fig. 37c) are filled with 3 ml of trichloroacetic acid each 0.16 ml, 0.17 ml or 0.18 ml of thymol bromine respectively, and 3 ml of Obermayer reagent (3). The tubes are left standing for four hours at room temperature, then centrifuged at high speed and the quantity of thymol bromide solution is noted which produces an oil droplet of 0.04 ml. Now an indican-series as shown in the pattern above is prepared, the titrated amount of thymol bromide is added and the tubes are allowed to stand for four hours. After centrifugation the liquid above the oil droplets is removed by suction and a layer of 6 ml of HCl ferric chloride reagent diluted twice with water is added.

### *Procedure*

*Quantitative determination of indican with the test tube.* Two-tenths of a milliliter (0.2) of whole blood or serum in 2.8 ml of distilled water is deproteinized with 1 ml of trichloroacetic acid (1) and filtered through a Whatman filter (Nr. 42). To 3 ml of filtrate in a tube as shown in fig. 37a the titrated amount of thymol (thymol bromide) is added and 3 ml of ferric chloride-HCl (3) well mixed and left four or twelve hours in a dark place. After that time the tube is centrifuged and compared with the tubes of the dilution series.

The amount of thymol (thymol bromide) required is determined according to the reaction time (four or twelve hours) and has to be titrated once with a blood sample, which has been treated as described above. The amount of thymol (thymol bromide) needed to produce a droplet of 0.04 ml varies between 0.16 and 0.19 ml for a reaction time of four hours, and between 0.11 and 0.14 ml for a reaction time of twelve hours. The titration must be repeated for each new lot of reagents.

A Walpole-comparator may be used for the estimation with openings corresponding to the lower part of the test tubes.

If more serum is available and it is desirable to work with a droplet size of 0.05 ml., the tubes are adjusted to that size and the amount of thymol is increased accordingly. The procedure is identical with the procedure described above except that 0.4 ml. of serum is diluted with 2.6 ml. of water.



FIG. 38. Test tube with insert for the colorimetric determination of indican in blood.

#### *Determination of Indican in the Hellige Colorimeter*

Reagents and procedure are identical with the above described method, only the test tubes have to be modified so that they will fit the Hellige-wedge. To this end the tubes are fitted as seen in figure 38. It is advisable to narrow the slot of the colorimeter so that the yellow acid-solution in the tube is invisible. A mixture of aqueous solutions of lithium carmine (C. ruebker) and methylene blue (Hoechst) to which a trace of NaF has been added is used for the standard. It is difficult to indicate exact directions for the preparation of a standard solution because the commercially available dye solutions are not uniform. If the correct dye mixture has once been established the standardization of the wedge is carried out with the aid of test tubes (figure 38) and an indican dilution series as described on p. 211. When plotted graphically the values must fall on a straight line.

Normal values: 0.026–0.082 mg. per cent. The nature of the diet is not significant. A physiologic hyperindicanemia is found during pregnancy (0.1–0.24 mg. per cent).

#### *Pathologic Hyperindicanemias*

(1) intestinal diseases, especially in cases of intestinal occlusion, constipation (rise up to 0.24 mg. per cent).

(2) kidney insufficiency all degrees of hyperindicanemia are encountered, values above 0.32 mg per cent are important for differential diagnosis. In uremic coma values up to 2.7 mg per cent have been noted

(3) eclampsia in the beginning of the disease a rapid rise up to 0.6 mg per cent, fast decline with the disappearance of the acute symptoms.

(4) nephritis of pregnancy values of 0.7-0.8 mg per cent are found even before the disease enters the serious stage. A further rise is noted with increased severity of the disease. In contrast to eclampsia a high indican level will persist for many weeks after delivery, even at times when the urea level has returned to normal

### XANTHOPROTEIN REACTION

*Principle of the method* The aromatic compounds are nitrated with nitric acid and the resulting nitrocompounds form a yellow brown solution in NaOH.

#### *Reagents*

- (1) 20 per cent trichloroacetic acid
- (2) concentrated  $\text{HNO}_3$  (specific gravity 1.4)
- (3) pure NaOH, 33 per cent

#### *Procedure*

Serum is mixed with equal amounts of 20 per cent trichloroacetic acid, and filtered. To the clear protein free filtrate one-fourth of its volume of concentrated  $\text{HNO}_3$  is added and heated to slow boiling for  $\frac{1}{2}$  minute. After cooling approximately  $\frac{1}{2}$  of the volume of NaOH (3) is added. In normal cases a yellow color appears, in pathologic cases a yellow brown to brown color is visible, which can easily be recognized with some practice especially when frequent normal determinations are carried out.

In kidney insufficiency high indican values and high xanthoprotein levels will tend to run parallel. Both indicate an increase of intestinal putrefaction products in serum and are based upon aromatic derivatives of tyrosine and tryptophane. In normal persons the serum level of these products is very low. It is increased in cases of kidney insufficiency and uremia, and these analytical results in

connection with urea determination and nonprotein nitrogen-determination are frequently used for differential diagnosis (heart and kidney diseases)

### UNDETERMINED NITROGEN

The total amount of 'undetermined nitrogen' which amounts to 5-10 mg per cent under physiologic conditions is found as the difference between the value for nonprotein nitrogen and the sum of all known other fractions. The 'undetermined nitrogen' is found mainly in the corpuscular elements and consists probably of hippuric acid nucleotides and histones. The undetermined nitrogen is increased in some cases of eclampsia, in advanced chronic nephritis with nitrogen retention (uremia). It has not been proved that the increase of any substance present in the "undetermined nitrogen" fraction is responsible for the toxic symptoms of eclampsia or uremia. Example for the calculation of 'undetermined nitrogen'

nonprotein nitrogen	31 mg per cent
urea nitrogen	14 mg per cent
aminoacid-nitrogen	0 mg per cent
polypeptid nitrogen	3 mg per cent
total creatinine-nitrogen (4.8 mg per cent creatinine)	1.8 mg per cent
Uric acid nitrogen (4.3 mg per cent uric acid)	1.3 mg per cent
Indican nitrogen	0
	<hr/>
	26.1 mg per cent

The difference between 31 mg per cent and 26.1 mg per cent = 4.9 mg per cent indicates the 'undetermined nitrogen' of the analyzed sample

### DETERMINATION OF THE DOUBLE (DIFFERENTIAL) NITROGEN<sup>15</sup>

If whole blood or serum is deproteinized with phosphomolybdic acid (phosphotungstic acid) and the same sample with trichloroacetic acid the nonprotein nitrogen values obtained by the trichloroacetic acid method will be higher than those obtained with phosphomolybdic acid

Phosphomolybdic acid precipitates native proteins and phosphomolybdic acid

peptones. Trichloroacetic acid precipitates native protein and albumoses, but not the peptones. Metaphosphoric acid precipitates only the native proteins, no albumoses or peptones<sup>14</sup>. (If metaphosphoric acid is used the digestion must first be performed over an asbestos plate, and only after the foaming has ceased may the digestion be carried out over a wire mesh between flask and flame. The precipitate is filtered through hardened filter paper.)

### Procedure

(1) determination of nonprotein nitrogen, using phosphomolybdic acid, see p. 144.

(2) determination of nonprotein nitrogen using trichloroacetic acid. Procedure as described for (1), only instead of 2 ml of phosphomolybdic acid 2 ml of 20 per cent trichloroacetic acid is used for deproteinizing.

Normally the difference amounts to 0-3 mg per cent. The difference is larger under pathologic conditions (nephritis, uremia). It has not yet been ascertained to which compounds the difference is due.

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- <sup>38</sup> SILBERSTEIN I RAFFAPORT F AND PININSKY, I : *Klin Wochenschr* 14 122 1936



## Chapter VI

# Proteins and Protein-Fractions

The nitrogen compounds in blood exist as proteins and compounds of non protein structure. The proteins in plasma are divided into albumins and globulins (euglobulin, pseudoglobulin, fibrinogen). During the process of clotting the fibrinogen is converted into fibrin, which forms the pulp of the blood clot.

### TITRIMETRIC DETERMINATION OF TOTAL PROTEINS IN SERUM OR PLASMA<sup>1</sup> (KJELDAHL METHOD)

*Principle of the method* Serum or plasma is digested with concentrated  $H_2SO_4$  in a micro-Kjeldahl flask and the nitrogen is determined titrimetrically in the residue.

#### *Reagents*

(See nonprotein nitrogen p. 144.)

#### *Procedure*

Two hundredths of a milliliter of serum or plasma is drawn in a long capillary pipet (Sahlb pipet), the tip is wiped carefully and the contents are delivered into a micro-Kjeldahl flask. The pipet is rinsed by running some distilled water through from the upper end. After the addition of 2 ml. of phosphomolybdic sulfuric acid and 2 glass beads the sample is digested. In order to prevent foaming of the mixture, the digestion has to be started very gradually heating the flask with a small flame and using an asbestos pad. With each series of determinations 2-3 blank determinations must be run. The remainder of the procedure is carried out as described for non protein nitrogen (p. 144).

#### *Calculation*

One ml. of N/100 thiosulfate corresponds to 0.0467 mg. of nitrogen. Thiosulfate used up by the blank minus thiosulfate used up by the sample multiplied by 233.5 results in nitrogen content of 100 ml.

of serum or plasma used for the analysis. The factor 233.5 is calculated as follows

$$\frac{0.0407 \text{ (mg. N)}}{0.02 \text{ (ml serum)}} \times 100 = 233.5$$

Total nitrogen content minus nonprotein nitrogen equals protein-nitrogen. The protein content of the sample is obtained by multiplication with 6.25 (see tables 10 and 11)

### Example

thiosulfate used up by blank.	0.80 ml
thiosulfate used up by sample	4.70 ml
difference	<hr/> 5.10 ml

$5.10 \times 233.5 = 1190.8$  mg per cent nitrogen. The value obtained for nonprotein nitrogen (p. 149) 230 mg per cent is deducted from the above figure resulting in the protein-nitrogen value of 1061.8 mg per cent. In order to find the protein content of the sample this figure is multiplied by 6.25 = 6640 mg per cent or 6.64 grams per cent protein in the sample.

Total protein determination with the copper-sulfate method (see p. 9)

In normal humans the serum (plasma) protein values vary between 6 and 8 mg per cent. Hyperproteinemia is found in\*

(a) insufficient fluid intake.

(b) fluid loss like intestinal obstruction and fistulas, diarrhea, vomiting, severe diabetic acidosis, intense heat exertion, Addison's disease, shock, burns, fulminant infections.

(c) diseases involving the reticulo-endothelial system (high globulin): multiple myeloma, monocytic leukemia, liver cirrhosis, cancer.

(d) chronic infections: ulcerative tuberculosis, syphilis, lymphopathia venereum, subacute bacterial endocarditis, periarthritis nodosa, lupus erythematosus, rheumatoid arthritis, Boeck's sarcoid, leprosy, kala-azar, schistosomiasis, filariasis, trypanosomiasis.

From the United States Navy Research Unit of the Rockefeller Institute for Medical Research.

TABLE 10—Table for the calculation of total nitrogen

Ml N/100  $\text{Na}_2\text{S}_2\text{O}_8$  correspond to mg% N if 0.02 ml. sample is used (see p 218) The table may also be used for the calculation of fibrinogen N if a division by 10 is done first.

Mg% N if a 0.003333 ml sample  $\left(\frac{0.2}{6.2}\right)$  is used.

	0	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.50	0.55	0.60	0.65	0.70	0.75	0.80	0.85	0.90	0.95
0	—	11.86	23.72	35.58	47.44	59.29	71.15	83.01	94.87	106.73	118.58	130.44	142.29	154.15	166.01	177.87	189.72	201.58	213.44	225.29
1	23.9	35.75	47.61	59.47	71.32	83.18	95.04	106.89	118.75	130.61	142.47	154.32	166.18	178.04	189.89	201.75	213.61	225.47	237.32	249.18
2	47.8	59.65	71.51	83.37	95.22	107.08	118.94	130.79	142.65	154.51	166.37	178.22	190.08	201.94	213.79	225.65	237.51	249.36	261.22	273.08
3	71.7	83.52	95.38	107.24	119.09	130.95	142.81	154.67	166.52	178.38	190.24	202.09	213.95	225.81	237.66	249.52	261.38	273.23	285.09	296.95
4	95.6	107.45	119.31	131.17	143.02	154.88	166.74	178.59	190.45	202.31	214.16	226.02	237.88	249.73	261.59	273.44	285.30	297.16	309.01	320.87
5	119.5	131.3	143.16	155.02	166.88	178.73	190.59	202.45	214.31	226.16	238.02	249.88	261.73	273.59	285.45	297.31	309.16	321.02	332.88	344.73
6	143.4	155.2	167.06	178.91	190.77	202.62	214.48	226.34	238.19	250.05	261.91	273.76	285.62	297.48	309.33	321.19	333.05	344.90	356.76	368.61
7	167.3	179.1	190.94	202.8	214.66	226.51	238.37	250.22	262.08	273.94	285.79	297.65	309.51	321.36	333.22	345.08	356.93	368.79	380.64	392.5
8	191.2	203	214.83	226.69	238.54	250.4	262.26	274.11	285.97	297.82	309.68	321.53	333.39	345.24	357.1	368.96	380.82	392.67	404.53	416.38
9	215.1	226.9	238.75	250.61	262.46	274.32	286.17	298.03	309.88	321.74	333.59	345.45	357.3	369.16	381.02	392.87	404.73	416.58	428.44	440.29
10	239	250.8	262.65	274.51	286.37	298.22	310.08	321.93	333.79	345.64	357.5	369.36	381.21	393.07	404.92	416.78	428.63	440.49	452.34	464.19

ml	0.81	0.82	0.83	0.84
mg	3.34	4.07	7.00	9.34
mg	1.44	3.33	4.34	5.70

TABLE 11.—Table for the calculation of protein  
1 mg of nitrogen corresponds to 6.25 mg. of protein

	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95
0	0	31.25	62.50	93.75	125.00	156.25	187.50	218.75	250.00	281.25	312.50	343.75	375.00	406.25	437.50	468.75	500.00	531.25	562.50	593.75
0	0	62.50	125.00	187.50	250.00	312.50	375.00	437.50	500.00	562.50	625.00	687.50	750.00	812.50	875.00	937.50	1000.00	1062.50	1125.00	1187.50
0	12.50	128.13	256.25	384.38	512.50	640.63	768.75	896.88	1025.00	1153.13	1281.25	1409.38	1537.50	1665.63	1793.75	1921.88	2050.00	2178.13	2306.25	2434.38
0	25.00	256.25	512.50	768.75	1025.00	1281.25	1537.50	1793.75	2050.00	2306.25	2562.50	2818.75	3075.00	3331.25	3587.50	3843.75	4100.00	4356.25	4612.50	4868.75
0	37.50	384.38	768.75	1153.13	1537.50	1921.88	2306.25	2690.63	3075.00	3459.38	3843.75	4228.13	4612.50	4996.88	5381.25	5765.63	6150.00	6534.38	6918.75	7303.13
0	50.00	500.00	1000.00	1500.00	2000.00	2500.00	3000.00	3500.00	4000.00	4500.00	5000.00	5500.00	6000.00	6500.00	7000.00	7500.00	8000.00	8500.00	9000.00	9500.00
0	62.50	625.00	1250.00	1875.00	2500.00	3125.00	3750.00	4375.00	5000.00	5625.00	6250.00	6875.00	7500.00	8125.00	8750.00	9375.00	10000.00	10625.00	11250.00	11875.00
0	75.00	750.00	1500.00	2250.00	3000.00	3750.00	4500.00	5250.00	6000.00	6750.00	7500.00	8250.00	9000.00	9750.00	10500.00	11250.00	12000.00	12750.00	13500.00	14250.00
0	87.50	875.00	1750.00	2625.00	3500.00	4375.00	5250.00	6125.00	7000.00	7875.00	8750.00	9625.00	10500.00	11375.00	12250.00	13125.00	14000.00	14875.00	15750.00	16625.00
0	100.00	1000.00	2000.00	3000.00	4000.00	5000.00	6000.00	7000.00	8000.00	9000.00	10000.00	11000.00	12000.00	13000.00	14000.00	15000.00	16000.00	17000.00	18000.00	19000.00

*Low protein values are found in*

(a) acute hemorrhage or chronic weeping wounds or skin lesions (burns), albuminuria, shock (surgical or traumatic)

(b) malnutrition low protein diet, vitamin deficiencies (beri beri, pellagra, etc.), incomplete absorption, sprue, cancer of stomach or pancreas, pernicious anemia, diabetes mellitus unregulated, hyperthyroidism, toxemia of pregnancy, especially in eclampsia.

(c) conditions in which albumin synthesis is retarded because of liver damage (low albumin) cirrhosis and cancer of liver, chronic poisoning

### TITRIMETRIC DETERMINATION OF FIBRINOGEN\* (ACCORDING TO THE KJELDAHL METHOD)

*Principle of the method* The fibrinogen present in citrated plasma is precipitated with calcium chloride, digested with  $H_2SO_4$  (phosphomolybdic acid as catalyst) and the nitrogen in the isolated fibrin is determined as described above.

*Reagents*

- (1) sterile 3.8 per cent sodium citrate solution. tribasic
- (2) 0.85 per cent sodium chloride solution
- (3) 1 per cent  $CaCl_2$  solution

*Procedure*

With a capillary pipet 0.1 or 0.2 ml of citrated plasma (1 part of sodium citrate and 9 parts of blood) is transferred to a test tube containing 5 ml of physiologic NaCl solution. The tip of the pipet must be carefully wiped off, and the pipet is rinsed by repeated sucking up and blowing out of the NaCl solution. After the addition of 1 ml of a 1 per cent  $CaCl_2$  solution the tubes are placed into an incubator of  $37^\circ C$  for 30 minutes to accelerate the precipitation. The tubes are allowed to cool to room temperature and the contents are filtered through glass wool\* (Before use the glass wool must be cleaned with cleaning solution and rinsed with water and hot physiologic NaCl solution). To remove the serum proteins the fibrin clot is washed 8-10 times with 2-3 ml of normal saline, during the washing the glass wool is squeezed with a small glass spatula. (If the

\* Coarse long threaded glass wool

nitrogen of fibrinogen free plasma is to be determined the filtrate is collected in a 25 ml. volumetric flask, the flask is made up to the mark with NaCl solution and the nitrogen is determined in 4 ml of filtrate) The fibrin flake and the glass wool are transferred to a micro-Kjeldahl flask with a pair of forceps and after the digestion with 2 ml of phosphomolybdic-sulfuric acid the procedure is continued as described above. A blank determination must be carried out together with each sample.

### Calculation

Thiosulfate required by the blank minus thiosulfate required by the sample multiplied by 46.7 results in the nitrogen content expressed in mg per cent, when 0.1 ml of plasma is used. If 0.2 ml. of plasma has been analysed, the factor is 23.35. To all results 10 per cent must be added, because 10 per cent citrate solution has been added to the blood sample. Fibrinogen nitrogen multiplied by 0.25 = fibrinogen-protein in mg per cent. (See tables 10 and 11)

### Example

thiosulfate used up by the blank	9.80 ml
thiosulfate used up by sample (0.2 ml plasma)	7.45 ml
difference	<u>2.35 ml</u>

$2.35 \times 23.35 = 54.87$  mg per cent fibrinogen nitrogen in citrated plasma

$54.87 \times 0.25 = 13.72$  mg per cent fibrinogen protein. As citrated plasma has been used 10 per cent must be added to this figure. Fibrinogen content of native undiluted plasma =  $377.3$  mg per cent =  $0.377$  Gm per cent protein

Fibrinogen content of normal plasma = 200-400 mg per cent.

The fibrinogen is increased in cases of slight liver damage (hepatitis) and in cases of acute tissue destruction, in most infectious diseases except typhoid fever during pregnancy and menstruation after X-ray treatment in cases of local infections (angina and cholecystitis) and in nephrosis.

The fibrinogen content decreases in liver insufficiency as fibrinogen is formed in the liver all cases of decreased liver function lead to a lowered fibrinogen level in plasma. Extremely low fibrinogen levels

are encountered in chloroform-phosphorus- and carbon tetrachloride poisoning and also in acute yellow atrophy of the liver Typhoid fever is one of the few infectious diseases where the fibrinogen level is lowered Low levels are also noted in acute loss of blood and in cachexia

## TITRIMETRIC DETERMINATION OF ALBUMIN AND GLOBULINS (KJELDAHL METHOD)

### *Reagents*

- (1) 22.2 per cent sodium sulfate solution
  - (2) 18 per cent sodium sulfate solution
  - (3) 14 per cent sodium sulfate solution
- For all other reagents see p 145

### *Preparation of the sodium sulfate solutions*

Sodium sulfate is freed from crystal water in a drying oven at 110°C and placed into a desiccator The solutions are made up with distilled water and kept in an incubator Before use the solutions are removed from the incubator and cooled down to room temperature without shaking the flasks If care is being taken in the preparation of the reagents the blank values for all fractions are identical and correspond to those of the phosphomolybdic sulfuric acid When all reagents are frequently checked it suffices to determine the blank value for phosphomolybdic-sulfuric acid only in the analysis of all protein fractions.

### *Procedure*

Six ml of 22.2 percent sodium sulfate solution are mixed with 0.2 ml of serum or citrated plasma (Final concentration of the sodium sulfate solution = 21.5 per cent) The pipet is rinsed by 3 times sucking up and blowing out of the liquid. The tubes are stopped and kept at 37° for 3 hours in order to complete the salting-out of the globulins and it is filtered at this temperature through a double filter (Whatman Nr 42) After cooling to room temperature 1 ml of the filtrate is transferred into a micro-Kjeldahlflask, 2 ml of phosphomolybdic-sulfuric acid is added the filtrate is digested carefully and the procedure continued as described above The filtrate con

tains albumin and nonprotein nitrogen. If albumin alone is to be determined the nonprotein nitrogen must be analyzed in a separate sample.

The following method describes a rapid separation of globulins from albumin.<sup>3</sup> To 6 ml of a 22.2 per cent solution of sodium sulfate contained in a wide centrifuge tube, 0.2 ml of serum is added. The capillary pipet is washed by sucking up and blowing out the mixture. After five minutes 2-3 ml of ether is added, the tubes stoppered and vigorously shaken for fifteen seconds. The tubes are allowed to stand a few minutes in the incubator at 37°C (or in a 40° water bath) and centrifuged. A ring of globulin is formed between sodium sulfate and ether. This ring is detached from the wall by holding the tube

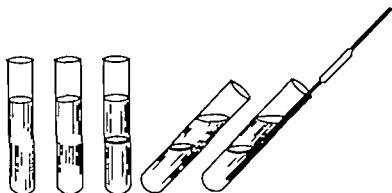


FIG. 39 Position of test tubes for albumin determination

in an oblique position. By introducing the pipet down to the bottom of the tube the clear fluid containing albumin can be removed (see fig. 39). This fluid can be obtained as well by filtration. Care must be taken in this procedure, that the sodium sulfate does not cool off during centrifugation, as crystallization will then occur and the  $\text{Na}_2\text{SO}_4$  concentration of the solution will decrease. This can be avoided by preheating the centrifuge cups with water of 40°C. Carbon tetrachloride may be used instead of ether but the following precautions must be taken. After the serum has been added to the  $\text{Na}_2\text{SO}_4$ , the mixture must be left in the incubator for 20 minutes, 2 ml of  $\text{CCl}_4$  are added, the tube is well shaken and again left in the incubator for 20 minutes until the layers have separated. After centrifugation the upper layer is used.



*Calculation*

Thiosulfate used up by the blank minus thiosulfate used up by the sample multiplied by 144.77 results in the nitrogen-content of the filtrate in mg per cent (albumin nitrogen + nonprotein nitrogen) One ml. of filtrate corresponds to 0.03223 ml of serum  $\left(\frac{0.2}{6.2}\right)$

One ml of N/100 thiosulfate corresponds to

$$\frac{0.0467 \text{ (mg N)}}{0.03223 \text{ (ml serum)}} \times 100 = 144.77 \text{ mg. in 100 ml. sample.}$$

*Example*

(See also tables 8 and 9)

albumin nitrogen + nonprotein nitrogen	840 mg per cent
nonprotein nitrogen	20 mg per cent
albumin nitrogen	811 mg per cent

$811 \times 0.25 = 5068.7$  mg. per cent or 5.07 g. per cent albumin protein.

Total nitrogen	1190.8 mg per cent
albumin nitrogen + non protein nitrogen	840.0 mg per cent
globulin nitrogen	350.8 mg per cent

$350.8 \times 0.25 = 2192$  mg per cent or 2.19 g per cent globulin protein.

If plasma is used for the analysis the fibrinogen will precipitate together with the globulin, and this value has to be deducted from the total protein when citrated plasma is used each figure has to be increased by 10 per cent (Dilution factor)

## DETERMINATION OF EUGLOBULIN (KJELDAHL METHOD)

Two tenths of a milliliter of serum or plasma is added to 6 ml of 14 per cent  $\text{Na}_2\text{SO}_4$  solution (a concentration of 13.5 per cent results) The sample is placed into an incubator ( $37^\circ\text{C}$ ) for three hours and treated as described above

*Calculation*

Thiosulfate used up for the blank minus thiosulfate used up for the sample multiplied by 144.77 equals nitrogen content of the fil-

trate in mg per cent. Total nitrogen less nitrogen of this filtrate equals euglobulin nitrogen (see tables 10 and 11)

#### DETERMINATION OF PSEUDOGLOBULINS I AND II (JAJELDAIL METHOD)

Two tenths of a milliliter of serum or plasma is added to 6 ml of 18 per cent  $\text{Na}_2\text{SO}_4$  solution ( $\text{Na}_2\text{SO}_4$  concentration of 17.4 per cent resulting). The tubes are placed into an incubator of  $37^\circ\text{C}$  for 3 hours and the filtrate is treated as described above. The difference between thiosulfate required by the blank and thiosulfate required by the sample multiplied by 144.77 gives the nitrogen content of this filtrate in mg per cent (see tables 10 and 11)

Pseudoglobulin I is calculated as the difference between the nitrogen-content of the 13.5 per cent filtrate and that of the 17.4 per cent filtrate. Pseudoglobulin II is calculated from the nitrogen-difference between the 17.4 per cent filtrate and the 21.5 per cent filtrate.

#### DIRECT TITRIMETRIC METHOD FOR THE DETERMINATION OF ALBUMIN IN SERUM AND PLASMA<sup>4</sup> (ACIDIMETRIC METHOD)

*Principle of the method* After removal of the globulins by means of sodium sulfate the protein solution (containing albumin only) is precipitated with a standardized phosphomolybdic acid solution. The excess of phosphomolybdic acid, having formed a complex compound with sodium oxalate, is titrated with sodium hydroxide, phenolphthalein being used as indicator. If  $\text{NaOH}$  and phenolphthalein only are used for the titration with phosphomolybdic acid the change of color is not distinct. The pink color disappears due to the buffer effect of the phosphoric acid contained by the phosphomolybdic acid, which forms mono-, di- and tri-sodium compounds. A persisting color can be obtained only by an excess of  $\text{NaOH}$  which is incompatible with quantitative exact titration. This difficulty can not be overcome by the use of another indicator. If however a sufficient amount of a sodium oxalate solution is added it forms sodium phosphates and the freed oxalic acid compound is easily titrated, giving a sharp change and a persisting color.

#### Reagents

- (1) 22.2 per cent  $\text{Na}_2\text{SO}_4$  aqueous solution
- (2) approximately 1 per cent phosphomolybdic acid aqueous solu

tion 2.5 ml of this solution should correspond to about 5 ml. of a N/25 NaOH

(3) 0.5 per cent alcoholic phenolphthalein solution

(4) approximately 1 per cent aqueous sodium oxalate solution

(5) N/25 sodium hydroxide! This solution must be kept on ice and should be freshly prepared about every week from a N/1 NaOH solution

(6) ether sulf pure

### *Procedure*

Two tenths of a milliliter of serum or plasma are placed with a capillary pipet into a wide centrifuge tube containing 6 ml of a 22.2 per cent solution of sodium sulfate (1). The solution is sucked up into the pipet and blown out to remove serum or plasma adhering to the inner wall of the pipet. This is repeated several times. After 5 minutes, 2-3 ml of ether<sup>1</sup> (6) are added, the tube is stoppered and shaken vigorously for fifteen seconds. It is allowed to stand for some minutes in the 37°C incubator (or in a 40° water bath) and centrifuged. The globulin ring, which forms, is detached from the wall by holding the tube in an oblique position (fig 38). By introducing the pipet down to the bottom of the tube the clear filtrate can be taken out. Three milliliters of phosphomolybdic acid (2) are placed into a dry wide test tube (Hagedorn-Jensen). While gently shaking the tube 3.1 ml (with a pipet marked at 3.0 and 3.1 ml) of the clear filtrate (corresponding to 0.1 ml of serum or plasma) are added drop by drop. The mixture is poured into a conical centrifuge tube and centrifuged for three minutes. Exactly 5 ml of supernatant are measured into a wide tube or flask and 2 ml of a 1 per cent solution of sodium oxalate (4) are added. The tubes are immersed for two minutes in a boiling water bath. While hot, the solution is titrated with N/25 NaOH (5) and phenolphthalein (3) as indicator until a persisting faint pink tint is obtained. As a blank 3 ml of phosphomolybdic acid are mixed with 3.1 ml of 22.2 per cent sodium sulfate. Five milliliter of this solution are used for the titration as above.

It is advisable to work with 2 controls and 3 blanks. All test tubes used must be washed with distilled water and care should be taken that they are absolutely dry.

*Calculation*

Number of milliliters of N/25 NaOH required for the titration of the analysis subtracted from those required for the blank and multiplied by the factor gives grams per cent albumin, (blank estimation)  $\times$  factor = Gm per cent albumin.

The factor is calculated by comparing a series of estimations according to the phosphomolybdic acid method with the Kjeldahl method

*Example*

Blank with phosphomolybdic acid	5 10 ml N/25 NaOH
Estimation with phosphomolybdic acid	4 18 ml. N/25 NaOH
difference	<hr/> 0 92 ml N/25 NaOH

The difference of 0 92 corresponds, e.g. to 4.5 Gm per cent albumin according to the Kjeldahl method  $\frac{4.5}{0.92} = 4.9$  A factor 4.9 results From several factors thus obtained the final factor is calculated

It is advisable to prepare sufficiently great quantities of phosphomolybdic acid and  $\text{Na}_2\text{SO}_4$  solution. (1-2 liters) because the factor remains constant only as long as the solutions are not changed The deviations of the factor are not very great (to approximately  $\pm 0.2$ ) for exact working however of importance

#### COMBINATION OF TITRIMETRIC AND COLORIMETRIC METHOD FOR THE DETERMINATION OF TOTAL PROTEINS ALBUMINS AND GLOBULINS

*Principle of the method* The total proteins are determined according to the cuppersulfate or the Kjeldahl method The albumin is determined colorimetrically with the biuret reaction (Copper sulfate + NaOH) the same serum serving as standard

*Reagents*

(1) standard serum serum the protein content of which has been determined with the cuppersulfate—or Kjeldahl method

- (2) 2 per cent NaOH
- (3) 1 per cent Coppersulfate solution
- (4) 8 per cent NaOH
- (5) 22.2 per cent sodiumsulfate solution
- (6) ether sulf

### Procedure

(1) Two tenths of a milliliter of serum and 2 ml. of  $\text{CuSO}_4$  solution (3) are added to 8 ml. of 2 per cent NaOH

(2) One milliliter of 8 per cent NaOH (4) and 1 ml. of  $\text{CuSO}_4$  (3) are added to 3.1 ml. (the 3 ml. pipet is marked at 3.1 ml.) of  $\text{Na}_2\text{SO}_4$  filtrate\* of the same serum

Tubes (1) and (2) are centrifuged and the supernatant of the tube containing the total proteins is filled into the wedge of the colorimeter and the supernatant containing the albumins is filled into the cup. Now the colors are compared.

### Example

Total proteins (Kjeldahl or coppersulfate method)	6.3 Gm. per cent
reading on the Hollige colorimeter	35

The serum contains 0.5 per cent albumins. The formula gives the content of albumins in grams per cent

$$\frac{6.3 \times 0.5}{100} = 4.09 \text{ Gm. per cent albumin}$$

The globulins are calculated as the difference between total proteins and albumin  $6.3 - 4.09 = 2.21$  Gm. per cent globulin. In a series of determinations a single serum of known protein-content may be used for the analysis of albumin, but if greater accuracy is desired, it is recommended to use as standard the same serum in which the albumin is to be determined.

The first indications of a disturbance in the protein level appear in a shift of the albumin:globulin ratio. The normal ratio of  $\frac{\text{albumin}}{\text{globulin}}$  varies between 1.6 and 2.5. If the albumin content is decreased, a

compensatory rise in globulin will occur and vice versa. An increase in the albumin fraction is extremely rare. A decreased albumin content, together with a rise in globulin is a most important clinical factor. It will occur in nephritis with extensive and lasting albuminuria also in many diseases which are accompanied with edema formation and ascites. The normal plasma content of albumin is 4.5-5.5 per cent and of globulin 1.5-3 per cent.

#### TITRIMETRIC DETERMINATION OF TOTAL PROTEINS IN SPINAL FLUID\* (KJELDAHL METHOD)

*Principle of the method* The total proteins of spinal fluid are precipitated with phosphomolybdic acid, centrifuged and the sediment after being dissolved in dilute sodium hydroxide, is quantitatively carried over to a Kjeldahl flask. The further procedures are those of nonprotein nitrogen determination (p. 143).

##### *Reagents*

##### (1) for protein precipitation

Five grams of anhydrous sodium sulfate, 4 Gm NaOH (pellets) and 80 ml. of phosphomolybdic acid are dissolved in 200 ml. of distilled water and the mixture boiled for one half hour. When the mixture is cool 10.6 ml. of concentrated  $H_2SO_4$  are added and the volume made up to 1000 ml.

##### (2) for digestion

One gram of phosphomolybdic acid is dissolved in about 50 ml. of water. One milliliter of 27 per cent sodium hydroxide is added and the mixture boiled for twenty minutes to drive off the possibly present ammonia whereupon 30 ml. of concentrated  $H_2SO_4$  and 5 ml. of syrupy phosphoric acid are added. The volume is made up with water to 100 ml. (If no phosphoric acid resistant digestion flasks are available no phosphoric acid may be added to the digestion mixture. A 10 per cent solution of  $Na_2HPO_4$  may be used instead, added after the digestion.)

##### (3) for neutralization

(a) 27 per cent solution of chemically pure NaOH (pellets)

(b) 15 mg. of methyl red and 60 mg. of thymol blue are dissolved in 20 ml. of N/1 NaOH and water is added up to 1000 ml.

(c) saturated solution of sodium fluoride (about 5 per cent)

The reagent ready for use is prepared by mixing 3 parts of solution

(a) with one half part of solution (b) and one and one half parts of solution (c) This mixture keeps indefinitely

(4) for titration

(a) buffer solution 84.5 Gm of boric acid and 15.6 Gm of sodium hydroxide (pellets) are dissolved in about 800 ml of water  $\text{NH}_3$  is removed by vigorous boiling for thirty minutes The solution is cooled and its volume made up to 1000 ml (instead of this solution a 10 per cent solution of  $\text{Na}_2\text{HPO}_4$  may also be used)

(b) 20 Gm of sodium bromide is dissolved in a little water in a measuring flask Eight Gm of bromine (= 2.5 ml) are added and the volume made up to 1000 ml Before use 10 ml. of this solution are poured into a 50 ml volumetric flask, diluted with 25 ml of buffer solution and made up to the mark with water

(c) potassium iodide crystals.

(d) fuming  $\text{HCl}$ , diluted with an equal volume of water

(e) 0.25 per cent starch solution

(f) N/100 sodium thiosulfate solution

### *Procedure*

One ml of centrifuged spinal fluid is placed in a small test tube (5 × 50 Widal tubes are best) If Pandy's reaction is positive only 0.5 ml of fluid is used Two milliliters of phosphomolybdic acid reagent are added to precipitate the proteins The tube is immersed in a 56°C water bath for a few minutes, then centrifuged for about ten minutes at 3000 rpm The supernatant fluid is carefully decanted and the sediment dissolved in 0.3 – 0.5 ml of 4 per cent sodium hydroxide The contents of the tube are now transferred carefully with a 1 ml serological pipet into a Kjeldahl flask, which contains 2 ml of digestion mixture (2) and a few glass beads or some glass wool The empty tube is filled again with some water, which is again transferred with a serological pipet to the Kjeldahl flask. This is repeated several times drawing up and blowing out the water in the pipet and washing the sides of the tube The micro-Kjeldahl flasks with the fluid to be analysed are set up, and similarly another two flasks are filled only with the digestion reagent as blanks Both series are boiled until the contents of the flasks become colorless again and fumes no longer arise The flasks are cooled and the residue diluted with 2–3 ml of water (or when the digestion mixture contains

no phosphoric acid the residue is diluted with 2 ml of 10 per cent  $\text{Na}_2\text{HPO}_4$ . The mixed indicator  $\text{NaOH}$ -sodium fluoride solution (3) is added drop by drop with continuous cooling with water. The solution being weakly acid is neutralized with 27 per cent  $\text{NaOH}$  solution until it turns blue. All flasks of a series of determinations require about the same number of drops. Five milliliters of hypobromite solution (4b mixed with 4a) are added to each flask which has been neutralized in the manner described above and are followed by a few crystals of  $\text{KI}$  and 2-3 ml of the solution of hydrochloric acid (4d). The titration is performed with  $\text{N}/100$  thiosulfate solution until yellow, and continued with starch indicator until colorless (see also photoelectric determination of protein in spinal fluid, p. 396).

### Calculation

1.0 ml of  $\text{N}/100$  thiosulfate equals 0.046 mg of nitrogen. If this figure is multiplied by the protein factor 6.25 we obtain 0.2875 mg of protein which multiplied by 100 gives the protein content in per cent.

### Example

2.1 ml of thiosulfate used. If 1 ml of spinal fluid is used we get  $2.1 \times 0.2875 \times 100 = 60.375$  mg per cent protein in the fluid. The quantity of thiosulfate used is calculated by subtracting the quantity used in the unknown from that in the blank.

In normal cases the protein content of spinal fluid varies between 15 and 40 mg per cent. It is elevated in all inflammatory processes of the brain in all organic changes or irritations caused by epilepsy or spastic states. In all these changes the albumin globulin ratio may vary as indicated by the course of the colloid reactions: gold or mastix-curves.

The protein content is elevated in <sup>7</sup>

tabes dorsalis	40-200 mg per cent
paralysis	30-190 mg per cent
lues cerebrospinalis	20-160 mg per cent
bacterial meningitis	160-1300 mg per cent
brain tumor	30-100 mg per cent (mostly negative mastix curve)
brain abscess	30-100 mg per cent
Froin syndrome	80-2100 mg per cent



ep encephalitis	50- 300 mg per cent
cerebral hemorrhage	20- 220 mg per cent
cerebral thrombosis	20- 140 mg per cent
epilepsy	15- 80 mg per cent
serous meningitis	20- 70 mg per cent

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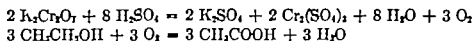
## Chapter VII

# Determination of Carbohydrates and Their Derivatives

### DETERMINATION OF ALCOHOL<sup>1</sup>

*Principle of the method* In the presence of sulfuric acid alcohol reduces potassium dichromate to chromic sulfate and the excess dichromate is determined iodometrically with thiosulfate

#### *Reaction equation*



#### *Reagents*

(1) Dichromate-sulfuric acid 0.2% Gm. of purest recrystallized potassium dichromate is dissolved in 1 ml of distilled water and made up to 100 ml with concentrated  $\text{H}_2\text{SO}_4$

(2) syrupy  $\text{H}_3\text{PO}_4$

(3) 5 per cent KI solution

(4) 0.2% per cent starch solution

(5) N/100 or N/200 sodium thiosulfate solution

#### *Apparatus*

(1) alcohol flask, consisting of two parts (see fig. 40)

(a) a 50 ml Erlenmeyer flask with ground-glass top

(b) ground glass stopper to fit the flask and carrying a small dish suspended on a glass rod. The length of the rod is such as to keep the dish 10–15 mm above the bottom of the flask when closed. Flask and stopper are equipped with hooks and springs to assure tight closure (see fig. 40)

(2) 0.1 ml capillary pipets with 2 marks, the lower one affixed approximately 30 mm above the tip. The marks are necessary to avoid evaporation of the alcohol by blowing out the blood.

(3) When no 1 ml automatic pipet is available (see append. p. 359)

the potassium dichromate reagent is measured out with a very long 1 ml pipet and allowed to flow slowly into the flask.

(4) incubator of 56-60°C

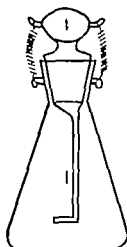


FIG. 40 Flask for alcohol determination according to Widmark.

### *Procedure*

Before drawing blood the Erlenmeyer flasks are filled with 1 ml of chromate-sulfuric acid each the acid is allowed to flow out very slowly (approximately 60 seconds) The accuracy of the method depends to a large extent upon the accurate measurement of the chromate-sulfuric acid If a series of determinations is to be performed the use of automatic pipets is recommended to assure an even flow and exact measurement of the reagent Blood is drawn from the finger tip into the above mentioned capillary pipet to the upper mark, the outside of the pipet is wiped off and the contents are allowed to flow slowly into the dish, until the blood reaches the lower mark The ground glass top is sealed with a drop of concentrated phosphoric acid The flask is closed, the stopper is secured with the springs and the samples (plus 3 blank runs) are placed into an incubator of 56-60°C for 2-3 hours After that time the flasks are removed, allowed to cool down to room temperature and opened Care must be taken that the dried blood powder does not drip into the acid Otherwise the sample must be discarded If the flask does not open readily the stopper can generally be removed by cooling the flask under running water The contents of all opened flasks are

diluted with exactly 20 ml of water, and cooled down 0.5 ml of potassium iodide solution is added and titration performed with N/100 thiosulfate from a microburette until a yellow color appears. Then a few drops of starch solution are added and the titration is continued till colorless. If small amounts of alcohol are expected, the titration is carried out with N/200 thiosulfate.

### Calculation

One ml of N/100 thiosulfate corresponds to 0.113 mg of alcohol (empirically determined for blood). The accurate factor is 0.115 calculated from the reaction equation which shows that 1 mol of alcohol corresponds to 2 atoms of oxygen or 4 equivalents. Consequently

$$1 \text{ ml of N/100 solution corresponds to } \frac{46}{4 \times 100 \times 1000} = 0.115$$

Thiosulfate used up by the blank minus thiosulfate used up by the sample multiplied by 113 gives the alcohol content in mg per cent, when 0.1 ml of blood and N/100 thiosulfate are used in the analysis.

### Example

thiosulfate used by the blank	3.85 ml
thiosulfate used by the sample	2.13 ml
difference	1.72 ml

The alcohol content is  $1.72 \times 113 = 194.36$  mg per cent. See also table 12 for calculation.

The reduction value of blood normally corresponds to 0.5 mg per cent of alcohol. The alcohol content of blood gives certain indications as to the degree of intoxication.

## LACTIC ACID DETERMINATION

### Original Method<sup>2</sup>

**Principle of the method** Blood is deproteinized with metaphosphoric acid and the carbohydrates quantitatively removed by copper sulfate-calcium hydroxide. Lactic acid is converted to acetaldehyde by heating with concentrated  $\text{H}_2\text{SO}_4$ . The intensity of the red color which appears upon addition of veratrol corresponds to the amount of acetaldehyde formed.

TABLE 12—Table for the calculation of alcohol

Ml N/100  $\text{Na}_2\text{S}_2\text{O}_3$  correspond to mg per cent alcohol

	0	1	2	3	4	5	6	7	8	9
0 0		1 13	2 26	3 39	4 52	5 05	6 78	7 91	9 04	10 17
0 1	11 30	12 43	13 56	14 69	15 82	16 95	18 08	19 21	20 34	21 47
0 2	22 00	23 73	24 86	25 99	27 12	28 25	29 38	30 51	31 64	32 77
0 3	33 00	35 03	36 16	37 29	38 42	39 55	40 68	41 81	42 94	44 07
0 4	45 20	46 33	47 46	48 59	49 72	50 85	51 98	53 11	54 24	55 37
0 5	56 50	57 63	58 76	59 89	61 02	62 15	63 28	64 41	65 54	66 67
0 6	67 80	68 93	70 06	71 19	72 32	73 45	74 58	75 71	76 84	77 97
0 7	79 10	80 23	81 36	82 49	83 62	84 75	85 88	87 01	88 14	89 27
0 8	90 40	91 53	92 66	93 79	94 92	96 05	97 18	98 31	99 44	100 57
0 9	101 70	102 83	103 96	105 09	106 22	107 35	108 48	109 61	110 74	111 87
1 0	113 00	114 13	115 26	116 39	117 52	118 65	119 78	120 91	122 04	123 17
1 1	124 30	125 43	126 56	127 69	128 82	129 95	131 08	132 21	133 34	134 47
1 2	135 60	136 73	137 86	138 99	140 12	141 25	142 38	143 51	144 64	145 77
1 3	146 90	148 03	149 16	150 29	151 42	152 55	153 68	154 81	155 94	157 07
1 4	158 20	159 33	160 46	161 59	162 72	163 85	164 98	166 11	167 24	168 37
1 5	169 50	170 63	171 76	172 89	174 02	175 15	176 28	177 41	178 54	179 67
1 6	180 80	181 93	183 06	184 19	185 32	186 45	187 58	188 71	189 84	190 97
1 7	192 10	193 23	194 36	195 49	196 62	197 75	198 88	200 01	201 14	202 27
1 8	203 40	204 53	205 66	206 79	207 92	209 05	210 18	211 31	212 44	213 57
1 9	214 70	215 83	216 96	218 09	219 22	220 35	221 48	222 61	223 74	224 87
2 0	226 00	227 13	228 26	229 39	230 52	231 65	232 78	233 91	235 04	236 17
2 1	237 30	238 43	239 56	240 69	241 82	242 95	244 08	245 21	246 34	247 47
2 2	248 60	249 73	250 86	251 99	253 12	254 25	255 38	256 51	257 64	258 77
2 3	259 00	260 13	261 26	262 39	264 42	265 55	266 68	267 81	268 94	270 07
2 4	271 20	272 33	273 46	274 59	275 72	276 85	277 98	279 11	280 24	281 37
2 5	282 50	283 63	284 76	285 89	287 02	288 15	289 28	290 41	291 54	292 67
2 6	293 80	294 93	296 06	297 19	298 32	299 45	300 58	301 71	302 84	303 97
2 7	305 10	306 23	307 36	308 49	309 62	310 75	311 88	313 01	314 14	315 27
2 8	316 40	317 53	318 66	319 79	320 92	322 05	323 18	324 31	325 44	326 57
2 9	327 70	328 83	329 96	331 09	332 22	333 35	334 48	335 61	336 74	337 87
3 0	339 00									

*Reagents*

(1) metaphosphoric acid, 10 per cent solution, prepared by dissolving purest grade metaphosphoric acid (acidum metaphosphoricum glaciale) in distilled water. Even if stored in the cold the solution will keep for 24 hours only.

(2) copper sulfate solution copper sulfate solution saturated in the cold is diluted 1:1 with water before use.

(3) calcium hydroxide (purest grade)

(4) veratrol solution 0.125 g veratrol (purest grade) is dissolved in 100 ml of abs alcohol

(5) Concentrated sulfuric acid c p If a greenish-yellow color appears within a few minutes after 0.1 ml of a 0.125 per cent alcoholic veratrol solution has been added to 3 ml. of  $H_2SO_4$  the acid is not suitable for use in this method

### *Procedure*

With an exactly calibrated capillary pipet 0.2 ml. of blood is drawn from the finger tip. In order to obtain blood without stasis a deep puncture must be made. If blood is drawn from the vein, no tourniquet must be used, the blood must be taken after complete muscle rest of at least 30 minutes.

*Deproteinizing* 0.2 ml of blood are delivered into 1.2 ml. of distilled water and the pipet rinsed 2-3 times by drawing up and blowing out the mixture. Then 0.2 ml of metaphosphoric acid are added and the tubes are shaken vigorously allowed to stand for about ten minutes and centrifuged at high speed. (The sulfosalicylic acid test in the supernatant must be negative)

To remove the carbohydrates, 0.8 ml of supernatant liquid is transferred into a conical centrifuge tube 0.2 ml of copper sulfate and 0.2 ml of calcium hydroxide are added and well mixed. The mixture is allowed to stand for thirty minutes, during this time it is stirred several times with a glass rod. Then the tubes are centrifuged at high speed and 0.5 ml of supernatant is removed with a capillary pipet (see fig. 4) care being taken not to remove copper hydroxide particles. The liquid is transferred to a dry tube. With cooling in ice water and continuous shaking 3 ml of sulfuric acid are added (or 0.25 ml of supernatant + 1.5 ml of sulfuric acid). The tubes are heated for exactly four minutes in a boiling water bath and immediately placed into ice water. After exactly two minutes 0.1 ml. or 0.05 ml of veratrol solution is added to the tubes, well mixed whereupon the tubes are placed into a waterbath of  $25^{\circ}C$  (a red color will appear when lactic acid is present). The colors are compared in the Hellgo colorimeter after exactly 20 minutes. In the case where 0.2 ml of sample and 1.5 ml of sulfuric acid have been used the cup of the colorimeter is too large and a glass wedge has to be inserted as described for the bilirubin determination (p. 323)

*Calculation*

The lactic acid content in mg per cent is read directly from the standardization curve of the test-wedge (see p 242)

### LACTIC ACID DETERMINATION WITH SIMULTANEOUS DEPROTEINIZATION AND REMOVAL OF SUGAR<sup>3 4</sup>

The lactic acid determination on blood according to Mendel-Goldscheider gives satisfactory results, but the method is cumbersome, when a whole series of determination has to be performed. The following method shortens and facilitates the procedure.

*Principle of the method.* Blood is deproteinized with copper sulfate-calcium hydroxide and the sugar is removed simultaneously. After centrifuging an aliquot of the clear supernatant is used for the lactic acid determination according to the method of Mendel-Goldscheider (Oxydation with sulfuric acid, and reading in a colorimeter after veratrol solution has been added.)

*Reagents*

- (1) 12.5 per cent copper sulfate solution
- (2) calcium oxyde c p
- (3) concentrated  $H_2SO_4$  (purest grade)
- (4) veratrol solution 0.125 Gm. of veratrol is dissolved in 100 ml. of abs. alcohol

*Procedure*

All tubes used for the reaction (Wassermann tubes, length 65 mm., diam 16 mm.) must be cleaned according to Mendel and Goldscheider. The tubes are filled with 2-3 ml. of concentrated  $H_2SO_4$  and the sulfuric acid is carefully diluted by filling the tubes up to the top with water. The dilute acid is left in the tubes until cooled down, then the tubes are rinsed till no more acid is present and left to dry. The required number of tubes (Wassermann tubes) is filled with 1.8 ml. of copper sulfate solution. (See fig 4.) With normal analytical precautions 0.2 ml. of blood is delivered from a capillary pipet. Then 0.4 Gm. of  $CaO$  is added to each tube and the contents are stirred with a thin glass rod until the mixture is homogenous and shows a dirty blue color. After standing for 1 hour the tubes are

centrifuged at high speed. One half (0.5) ml. of supernatant are transferred to a clean dry test tube and 3 ml of sulfuric acid are added with cooling in ice water and shaking. Then the tubes are heated in a boiling water bath for four minutes and left in icewater for two minutes, 0.1 ml. of veratrol solution is added to each tube. After twenty minutes at room temperature the colors are compared in the Hellige colorimeter.

### *Calculation*

The lactic acid content in mg per cent is read off from the standardization curve of the test wedge (see below).

This method is suitable only for the determination of lactic acid in blood directly drawn from the punctured finger tip (no fluoride blood).

### *Preparation and standardization of the wedge*

#### *Reagents*

(1) dilute aqueous solution of cobaltic sulfate

(2) dilute aqueous solution of orange G

(3) lactic acid solution 3.3 mg of lithium lactate dried to constant weight in a desiccator and corresponding to 50 mg of lactic acid are dissolved in 1000 ml of distilled water. A series of dilutions as shown in table 13 is prepared from the lactate solution. To each 0.5 ml. of the dilutions 3 ml of sulfuric acid and 0.1 ml of veratrol solution are added and further treated as described above. The tubes are finally compared in the colorimeter with a solution prepared by adding a few drops of orange G solution to the cobaltic sulfate solution. A graph is made, with the scale of the colorimeter marked on the ordinate, and the mg per cent lactic acid on the abscissa.

The normal lactic acid content of venous blood (no stasis) is 10-12 mg per cent.

An increased lactic acid content is found in

(1) increased muscle activity. The rise is less in a person used to exercise (20-60 mg per cent) than in a person not used to it (up to 140 mg per cent).

(2) pneumonia

(3) diseases of the lung accompanied by dyspnea

(4) heart diseases



(5) ether anaesthesia

(6) hypoglycemic shock. If such doses of insulin are injected as may lead to hypoglycemia, the lactic acid level rises, but this must not be regarded as a specific effect of the insulin upon the carbohydrate metabolism, it is rather caused by increased muscle activity during shock.

(7) pregnancy, especially in toxic pregnancy

(8) anemia

(9) some liver diseases

TABLE 13

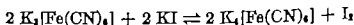
ml. lactate	ml. water	mg. % lactic acid
1	9	5
2	8	10
3	7	15
4	6	20
5	5	25
6	4	30
7	3	35
8	2	40
9	1	45
10	0	50

\* As standard and sample have been diluted 10 times.

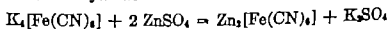
## BLOOD SUGAR

### *Method According to Hagedorn-Jensen\**

*Principle of the method.* The protein free blood filtrate is heated with an alkaline potassium ferricyanide solution, whereby the sugar reduces the ferric cyanide to ferrocyanide. After cooling the excess ferriocyanide is determined iodometrically according to the equation



However, this reaction is reversible. In order to get quantitative conversion the ferrocyanide must be precipitated by zinc sulfate as insoluble zinc ferrocyanide



\* Mainly according to H. Hagedorn and B. N. Jensen. *Biochem Zeitschr* 135 46 1923 137 92 1923

*Reagents*

(1) N/10 NaOH

(2) 0.45 per cent zinc sulfate solution prepared by diluting a 45 per cent zinc sulfate stock solution 100 times.

(3) N/200 potassium ferricyanide In a 1 liter volumetric flask 1.65 Gm. of potassium ferricyanide and 10.6 g. dried  $\text{Na}_2\text{CO}_3$  are dissolved in distilled water and made up to the mark with water. This solution must be stored in a dark bottle. If a large number of determinations is to be performed it is recommended to prepare the solution as follows:

(a) 16.5 Gm. of potassium ferricyanide (purest grade) is dissolved in 1000 ml. of distilled water.

(b) 106 g.  $\text{Na}_2\text{CO}_3$  (anhydrous) are dissolved in 1000 ml. of distilled water.

To prepare the working solution 100 ml. of solution (a) are mixed with 100 ml. of solution (b) and made up to 1 liter.

(4) 50 Gm. of zinc sulfate are dissolved in distilled water in a 1 liter volumetric flask. After the salt has dissolved 40 ml. of glacial acetic acid are added and made up to the mark with water. Before use, a 2.5 per cent solution of KI in this zinc sulfate solution is made up, i.e. 0.25 Gm. of KI for each 10 ml. of acid  $\text{ZnSO}_4$  solution.

(5) 0.25 per cent starch solution (see appendix p. 370)

(6) N/200 sodium thiosulfate solution

*Procedure*

A number of test tubes are filled with 1 ml. of NaOH (1) and 5 ml. of zinc sulfate solution (2) each and stoppered. The tubes, thus prepared, may be kept on hand as the mixture will keep indefinitely.

One tenth (0.1) ml. of blood is drawn from the finger tip with a capillary pipet and delivered into the above prepared zinc-hydroxide solution. The pipet is rinsed by repeated drawing up and blowing out the mixture. Some of the mixture together with a few air bubbles is then drawn into the pipet and slowly blown into the tubes. In this manner the pipet is rinsed quantitatively and at the same time dried so that the same pipet may be used for one or more control samples.

The samples (together with 3 blank determinations) are placed into a boiling water bath for three minutes, *this must be done imme-*

*diately to avoid glycolysis* After cooling, the samples are filtered through small funnels (diameter 3-4 cm) into wide tubes, (Hagedorn Jensen tubes, 30 x 90 mm) A small plug of cotton, washed previously with hot water is placed into the funnel to serve as filter The tubes are rinsed twice with 3 ml of boiling water and the wash water is also filtered Funnels and tubes may be washed with an

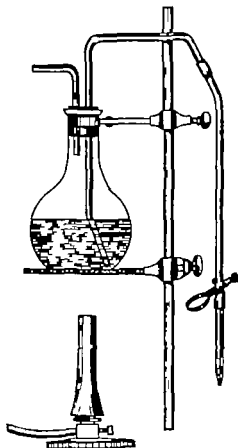


FIG 41 WATER CONTAINER WITH SYPHON

apparatus shown in figure 41 The filters are allowed to drain finally the cotton plug is squeezed out with a thin glass rod to obtain quantitative recovery of the filtrate The cotton should be kept in tightly closed glass jars equipped with ground glass stoppers, in order to avoid contamination with volatile reducing substances the cotton must also be fat-free. This is ascertained by placing a thin layer of cotton into a cylinder containing water Fat-free cotton will immediately sink to the bottom

To the protein-free filtrate 2 ml of alkaline potassium ferricyanide solution are added using an automatic pipet (appendix p 359) If no automatic 2 ml pipet is available, an exactly calibrated regular pipet is used The accuracy of the determination depends largely upon the exact measurement of the ferricyanide solution

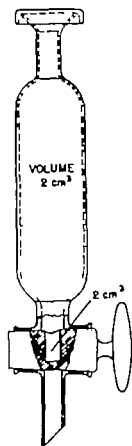


FIG 42 AUTOMATIC FILLER FOR ACID ZINC-SULFATE POTASSIUM IODIDE SOLUTION

Now all tubes are heated for fifteen minutes in a boiling water bath, or in cases where the result of the determination is urgently needed (in cases of coma) they are placed for three minutes into a glycerin bath at  $125^{\circ}\text{C}$  \* After cooling 2 ml of the acid zinc sulfate-potas-

According to the physical law a rise in temperature of 10 degrees C speeds up the reaction by about 2½ times (see cholesterol p 297) T Rappaport and F Eichhorn *Acta Med Orientalia* 11 4, 1913

sium iodide solution (4) (possibly using an automatic filler as shown in fig 42) are added. The liberated iodine is titrated with N/200 sodium thiosulfate solution, using a few drops of starch indicator towards the end of the titration. The sugar values are read from table 14. The difference of the sugar values between blank and sample gives mg of glucose in 0.1 ml of blood. Multiplication of this figure by 1000 results in glucose expressed in mg per cent.

TABLE 14 — *Table for the determination of glucose in blood according to Hagedorn-Jensen*

All N/200  $\text{Na}_2\text{S}_2\text{O}_3$  = mg glucose

	0	1	2	3	4	5	6	7	8	9
0 0	0 385	0 382	0 379	0 376	0 373	0 370	0 367	0 364	0 361	0 358
0 1	0 355	0 352	0 350	0 348	0 345	0 343	0 341	0 338	0 336	0 333
0 2	0 331	0 329	0 327	0 325	0 323	0 321	0 318	0 316	0 314	0 312
0 3	0 310	0 308	0 306	0 304	0 302	0 300	0 298	0 296	0 294	0 292
0 4	0 290	0 288	0 286	0 284	0 282	0 280	0 278	0 276	0 274	0 272
0 5	0 270	0 268	0 266	0 264	0 262	0 260	0 259	0 257	0 255	0 253
0 6	0 251	0 249	0 247	0 245	0 243	0 241	0 240	0 238	0 236	0 234
0 7	0 232	0 230	0 228	0 226	0 224	0 222	0 221	0 219	0 217	0 215
0 8	0 213	0 211	0 209	0 208	0 206	0 204	0 202	0 200	0 199	0 197
0 9	0 195	0 193	0 191	0 190	0 188	0 186	0 184	0 182	0 181	0 179
1 0	0 177	0 175	0 173	0 172	0 170	0 168	0 166	0 164	0 163	0 161
1 1	0 159	0 157	0 155	0 154	0 152	0 150	0 148	0 146	0 145	0 143
1 2	0 141	0 139	0 138	0 136	0 134	0 132	0 131	0 129	0 127	0 125
1 3	0 124	0 122	0 120	0 119	0 117	0 115	0 113	0 111	0 110	0 108
1 4	0 106	0 104	0 102	0 101	0 099	0 097	0 095	0 093	0 092	0 090
1 5	0 088	0 086	0 084	0 083	0 081	0 079	0 077	0 075	0 074	0 072
1 6	0 070	0 068	0 066	0 065	0 063	0 061	0 059	0 057	0 056	0 054
1 7	0 052	0 050	0 048	0 047	0 045	0 043	0 041	0 039	0 038	0 036
1 8	0 034	0 032	0 031	0 029	0 027	0 025	0 024	0 022	0 020	0 019
1 9	0 017	0 015	0 014	0 012	0 010	0 008	0 007	0 005	0 003	0 002

### Example

thiosulfate used up by the blank

1.93 ml

thiosulfate used up by the sample

1.08 ml

in table 14 1.08 ml of thiosulfate correspond to 0.163 mg sugar and 1.93 ml of thiosulfate correspond to 0.012 mg sugar. The difference multiplied by 100 gives a sugar content of 151 mg per cent.

The Hagedorn-Jensen method gives accurate values only up to 340 mg per cent of sugar. The accuracy cannot be increased by the use of larger quantities of potassium ferricyanide because the Hagedorn-Jensen reduction values do not follow a linear equation but form a parabolic curve. The curve shows that the calculation factor becomes less and less favorable with increasing sugar values.

*Method according to Fuyuta Akaji and Danzo Iwatake<sup>4</sup>*

**Principle of the method** The reduction of the potassium ferricyanide solution by sugar depends upon the pH. Under optimal conditions (buffering with phosphate) a linear blood sugar curve can be obtained. On account of the buffering the iodometric determination of excess ferric cyanide is carried out in mineral-acid solution.

*Reagents*

- (1) N/10 NaOH
- (2) 0.45 per cent zinc sulfate solution
- (3) (a) phosphate buffer 70 Gm. of secondary potassium phosphate and 21.25 Gm. of tertiary potassium phosphate are dissolved in water and made up to 1 liter in a volumetric flask.
- (b) 3.3 Gm. of potassium ferricyanide are dissolved in distilled water and made up to 1 liter. Before use, equal parts of solutions (a) and (b) are mixed.
- (4) 150 ml. of fuming HCl is diluted to 1000 ml. with distilled water.
- (5) 50 Gm. of zinc sulfate and 25 Gm. of KI are dissolved in re-distilled water and made up with water to 1000 ml.
- (6) 0.25 per cent starch solution.
- (7) N/200 sodium thiosulfate solution.

If only occasional analyses are to be performed it is recommended to prepare solutions (4) and (5) as follows: (4a) 50 Gm. of zinc sulfate are dissolved in 1000 ml. of water. Before use enough KI is added to give a 2.5 per cent solution of KI in  $\text{ZnSO}_4$ .

*Procedure*

Drawing of blood, deproteinizing and filtration are carried out as described for the original Hagedorn-Jensen method. To the clear filtrate 4 or 6 ml. of potassium ferricyanide-phosphate mixture are

added and the tubes are heated for fifteen minutes in a boiling water bath. After cooling 2 ml. of potassium iodide mixture and 2 ml. of hydrochloric acid are added to each tube, (for a series of determinations the use of an automatic filling device as shown in fig. 42 is recommended) and the liberated iodine is titrated with N/200 thiosulfate, using starch as indicator.

### Calculation

Thiosulfate used for the blank minus thiosulfate used for the sample multiplied by 0.174 equals sugar content of the sample in mg. If 0.1 ml. of blood has been analyzed, multiplication by 174 will give the sugar content in mg. per cent (see also table 15).

### Example

thiosulfate used for the blank	3.85 ml
thiosulfate used for the sample	2.91 ml
difference	0.94 ml

0.94 ml  $\times$  0.174 = a sugar content of 0.16350 mg. in 0.1 ml. of blood, or 163.50 milligrams per cent.

### *Titrimetric Determination of Sugar in 0.02 ml. of Whole Blood (Plasma, Serum)\**

In some cases it is difficult to obtain even 0.1 ml. of blood. The following method permits the determination of sugar in 0.02 ml. of sample. This method is convenient for series of determinations in infants and small animals, like rats, guinea pigs, and in the aqueous humor of the eye.

**Principle of the method** A known amount of potassium ferricyanide is reduced by sugar with heating and the excess ferricyanide is determined iodometrically.

### Reagents

- (1) N/50 NaOH
- (2) 0.45 per cent zinc sulfate solution prepared from a 45 per cent stock solution.
- (3) potassium ferricyanide-phosphate mixture (a) in a 1000 ml.

$$\text{MU N/200 or N/1000 Na}_2\text{S}_2\text{O}_3 = \text{mg \% glucose}$$

	0	1	2	3	4	5	6	7	8	9
0 0		1 74	3 48	5 22	6 96	8 70	10 44	12 18	13 92	15 66
0 1	17 40	19 14	20 88	22 62	24 36	26 10	27 84	29 58	31 32	33 06
0 2	34 80	36 54	38 28	40 02	41 76	43 50	45 24	46 98	48 72	50 46
0 3	52 20	53 94	55 68	57 42	59 16	60 90	62 64	64 38	66 12	67 86
0 4	69 60	71 34	73 08	74 82	76 56	78 30	80 04	81 78	83 52	85 26
0 5	87 00	88 74	90 48	92 22	93 96	95 70	97 44	99 18	100 92	102 66
0 6	104 40	106 14	107 88	109 62	111 36	113 10	114 84	116 58	118 32	120 06
0 7	121 80	123 54	125 28	127 02	128 76	130 50	132 24	133 98	135 72	137 46
0 8	139 20	140 94	142 68	144 42	146 16	147 90	149 64	151 38	153 12	154 86
0 9	156 60	158 34	160 08	161 82	163 56	165 30	167 04	168 78	170 52	172 26
1 0	174 00	175 74	177 48	179 22	180 96	182 70	184 44	186 18	187 92	189 66
1 1	191 40	193 14	194 88	196 62	198 36	200 10	201 84	203 58	205 32	207 06
1 2	208 80	210 54	212 28	214 02	215 76	217 50	219 24	220 98	222 72	224 46
1 3	226 20	227 94	229 68	231 42	233 16	234 90	236 64	238 38	240 12	241 86
1 4	243 60	245 34	247 08	248 82	250 56	252 30	254 04	255 78	257 52	259 26
1 5	261 00	262 74	264 48	266 22	267 96	269 70	271 44	273 18	274 92	276 66
1 6	278 40	280 14	281 88	283 62	285 36	287 10	288 84	290 58	292 32	294 06
1 7	295 80	297 54	299 28	301 02	302 76	304 50	306 24	307 98	309 72	311 46
1 8	313 20	314 94	316 68	318 42	320 16	321 90	323 64	325 38	327 12	328 86
1 9	330 60	332 34	334 08	335 82	337 56	339 30	341 04	342 78	344 52	346 26
2 0	348 00	349 74	351 48	353 22	354 96	356 70	358 44	360 18	361 92	363 66
2 1	365 40	367 14	368 88	370 62	372 36	374 10	375 84	377 58	379 32	381 06
2 2	382 80	384 54	386 28	388 02	389 76	391 50	393 24	394 98	396 72	398 46
2 3	400 20	401 94	403 68	405 42	407 16	408 90	410 64	412 38	414 12	415 86
2 4	417 60	419 34	421 08	422 82	424 56	426 30	428 04	429 78	431 52	433 26
2 5	435 00	436 74	438 48	440 22	441 96	443 70	445 44	447 18	448 92	450 66
2 6	452 40	454 14	455 88	457 62	459 36	461 10	462 84	464 58	466 32	468 06
2 7	469 80	471 54	473 28	475 02	476 76	478 50	480 24	481 98	483 72	485 46
2 8	487 20	488 94	490 68	492 42	494 16	495 90	497 64	499 38	501 12	502 86
2 9	504 60	506 34	508 08	509 82	511 56	513 30	515 04	516 78	518 52	520 26
3 0	522 00	523 74	525 48	527 22	528 96	530 70	532 44	534 18	535 92	537 66
3 1	539 40	541 14	542 88	544 62	546 36	548 10	549 84	551 58	553 32	555 06
3 2	556 80	558 54	560 28	562 02	563 76	565 50	567 24	568 98	570 72	572 46
3 3	574 20	575 94	577 68	579 42	581 16	582 90	584 64	586 38	588 12	590 86
3 4	591 00	593 34	595 08	596 82	598 56	600 30	602 04	603 78	605 52	607 26
3 5	609 00	610 74	612 48	614 22	615 96	617 70	619 44	621 18	622 92	624 66
3 6	626 40	628 14	629 88	631 62	633 36	635 10	636 84	638 58	640 32	642 06
3 7	643 80	645 54	647 28	649 02	650 76	652 50	654 24	655 98	657 72	659 46
3 8	661 20	662 94	664 68	666 42	668 16	669 90	671 64	673 38	675 12	676 86
3 9	678 60	680 34	682 08	683 82	685 56	687 30	689 04	690 78	692 52	694 26
4 0	696 00									



volumetric flask 0.9 Gm of potassium ferricyanide is dissolved in water and made up to the mark (b) phosphate buffer 21.0 Gm of secondary potassium phosphate and 63.75 Gm of tertiary potassium phosphate are dissolved in water and made up to 1 liter with water. Before use equal parts of solutions (a) and (b) are mixed.

(4) zinc sulfate potassium iodide reagent (a) to a 20 per cent zinc sulfate solution prepared from purest grade  $\text{ZnSO}_4$  in redistilled water are added immediately before use enough KI crystals to give a 2.5 per cent solution. This reagent may only be used on the day it is prepared.

(5) 20 per cent phosphoric acid, prepared by diluting 20 parts (by volume) of syrupy phosphoric acid with 80 parts (by volume) of water.

(6) N/1000 sodium thiosulfate solution. In a 1000 ml volumetric flask 12 ml of N/1 NaOH and 10 ml of N/10 sodium thiosulfate are placed and made up to the mark with distilled water. This solution will keep for 2-3 weeks.

(7) 0.25 per cent starch solution

### *Procedure*

The required number of test tubes (Wassermann tubes with hp, length 65 mm, diameter, 16 mm) plus 2-3 tubes for blank runs are filled with 1 ml of NaOH (1) each. With an exactly calibrated capillary pipet (Sahlb pipet with long tube) 0.02 ml of whole blood (plasma, serum) is drawn. The outside of the pipet is carefully wiped clean the blood is blown into the alkali and the pipet rinsed by drawing up and blowing out the mixture, the rinsing is repeated twice more. To all tubes 1 ml of zinc sulfate solution (2) is now added and the tubes are immersed in a boiling water bath for three minutes. (It is necessary to apply this method of deproteinizing—adding the blood to the alkali, to cause hemolysis and homogenizing and subsequent precipitation with zinc sulfate—for the following reason: very small amounts of blood added to colloidal zinc hydroxide coagulate the protein and enclose sugar in the particles, resulting in irregular and low sugar values.) After cooling to room temperature the mixture is filtered through a small funnel (diameter, 25 mm, the stem should be narrowed near the top) into Hagedorn-Jensen tubes. A small piece of cotton washed with hot water serves as filter. For the washing the use of the apparatus as shown on p 244, fig 41 is recommended. (The cotton must be stored in tightly closed tin cans or

glass jars.) The small test tubes are rinsed 3 times with 1 ml. of hot water each and the wash water is also filtered. The filters are allowed to drain finally the cotton plug is compressed with a thin glass rod to obtain quantitative recovery of the filtrate. To each tube 2 ml. of phosphate buffer-ferricyanide solution (3) are now added and the tubes are placed in a boiling water bath for twenty minutes to accomplish the reduction. After cooling 1 ml. of zinc sulfate-KI

TABLE 16

ml. of 0.0005 N Thiosulfate	Tenth of 1 ml. of 0.0005 N Sodium Thiosulfate									
	0	1	2	3	4	5	6	7	8	9
	Mg of glucose in 100 cc. blood									
0	—	—	21	23	26	29	31	34	36	39
1	41	44	46	49	51	53	56	58	61	63
2	65	68	70	72	75	77	80	82	84	86
3	89	92	94	97	99	101	103	106	108	110
4	113	115	117	119	121	124	126	128	130	132
5	135	137	139	141	143	146	148	150	152	154
6	157	159	161	163	165	168	170	172	174	176
7	179	181	183	185	187	190	192	194	196	199
8	201	203	205	207	210	212	214	216	218	221
9	223	225	227	230	232	234	237	239	241	243
10	245	248	250	252	254	256	259	261	263	265
11	267	270	272	274	276	279	281	283	285	288
12	290	292	294	296	299	301	303	305	308	310
13	312	314	316	318	321	323	326	328	330	332
14	334	337	339	341	343	345	347	350	352	354
15	356	359	361	363	365	367	370	372	374	376
16	378	381	383	386	388	390	392	394	396	398
17	400	—	—	—	—	—	—	—	—	—

solution (4) and 1 ml. of phosphoric acid solution (5) are added and titrated to colorless with N/1000 thiosulfate (6) from a micro-burette with a few drops of starch solution (7) as indicator. The color change is sharp even though a very dilute thiosulfate solution is used.

### Calculation

Thiosulfate used up by the blank minus thiosulfate used up by the sample multiplied by 174 gives the sugar content in mg. per cent (see also table 16)

*Example*

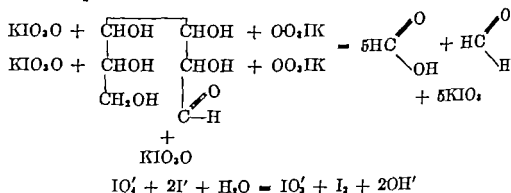
thiosulfate used up by the blank	2.30 ml
thiosulfate used up by the sample	1.62 ml
difference	0.68 ml

$$0.68 \text{ ml} \times 174 = 118.32 \text{ mg per cent sugar}$$

*Titrimetric Method for the Determination of Sugar in 0.01 or 0.005 ml. of Whole Blood, (Serum, Plasma)<sup>7</sup>*

Testing and evaluation of many hormone- and vitamin-preparations as well as many other problems require series of determinations in small animals where the exact estimation of sugar in very small amounts of blood is needed. The following periodate method affords the accurate determination of sugar in 0.01 ml resp 0.005 ml. of blood.

*Principle of the method* Sugar is oxydized in acid solution by periodate in a water bath, the periodate is reduced to iodate, and the excess periodate is determined iodometrically.

*Reaction equation**Reagents*

## A. for deproteinizing

(1) sodium silicate-phosphoric acid reagent To 166 ml. of 10 per cent sodium silicate solution ( $\text{Na}_2\text{SiO}_3 \times 8 \text{H}_2\text{O}$ , purest grade) in a 1 liter flask are added 7 Gm. of  $\text{NaCl}$ , 670 ml. of distilled water and 91 ml. of molar phosphoric acid, the flask is placed in a hot water bath until a distinct opalescence is noted. Then 66 ml. of molar phosphoric acid are added to the hot solution and the reagent is well mixed. When cool it is ready for use.

(2) 0.1 N NaOH.

(B) for the sugar determination

(3) acid periodate solution (a) in a 1000 ml volumetric flask 0.4 g of crystalline potassium periodate (purest grade) is dissolved in water with heating and after cooling made up to the mark with water (b) 10 per cent  $H_2SO_4$  (5.8 ml of  $H_2SO_4$  specific gravity 1.84 is made up to 100 ml with water)

Before use equal parts of solutions (a) and (b) are mixed

(4) 12 per cent solution of secondary potassium phosphate in distilled water

(5) potassium iodide.

(6) freshly prepared 0.001 N *neutral* sodium thiosulfate solution.

(7) 0.25 per cent starch solution

### *Procedure*

The required amount of test tubes (Wassermann tubes, length 65 mm diameter 16 mm) plus 3 extra tubes for blank runs are filled with 1 ml of sodium silicate-phosphoric acid reagent (1) and 0.8 ml. of 0.1 N NaOH (2) each. With an exactly calibrated and graduated Sahli capillary pipet\* 0.01 ml of whole blood (serum plasma) is measured out and blown out into the deproteinizing mixture. The pipet is rinsed 3 times by drawing up and blowing out the mixture. The tubes are well shaken and immersed in a boiling water bath for 3 minutes. After cooling to room temperature the contents of the tubes are filtered through small funnels into Hagedorn-Jensen tubes, a small piece of cotton (washed with hot water) serving as filter (see fig. 41). The tubes are rinsed 3 times with 1 ml. of hot water each and the wash water is also filtered. The Hagedorn-Jensen tubes must be washed thoroughly before use and rinsed with distilled water. The last drops of fluid are squeezed out of the cotton plug with a thin glass rod. To each tube 2 ml of acid periodate solution (3) are added and the tubes placed into a boiling water bath for twenty minutes to accomplish the reduction. After cooling 4 ml of secondary phosphate solution (4) are added and well mixed. Now a few crystals of KI (5) are added and after repeated mixing the liberated iodine is titrated from a micro-burette with 0.001 N thiosulfate solution (6). Several drops of starch solution (7) are added as indicator.

\* See appendix p. 361

*Calculation*

Thiosulfate used up by the blank minus thiosulfate used up by the sample multiplied by 190 gives the sugar content of the sample in mg per cent (if only 0.005 ml of blood were used, the difference has to be multiplied by 380)

*Semimicro-Determination of Blood Sugar according to Shaffer and Hartmann<sup>2</sup>*

*Principle of the method* The alkaline copper (3) reagent is reduced to copper (2) oxide by sugar in the heat. After cooling and acidifying iodine is liberated from  $\text{KIO}_3$  and  $\text{KI}$ , contained in the reagent, which in turn oxidizes  $\text{Cu}_2\text{O}$  to  $\text{CuO}$ . From the titrimetric determination of the excess iodine, the iodine used up for the oxidation of  $\text{Cu}_2\text{O}$  to  $\text{CuO}$  can be estimated. From this figure the amount of sugar present in the sample can be calculated.

*Reagents*

- (1) 10 per cent sodium tungstate solution
- (2)  $\frac{2}{3}$  N  $\text{H}_2\text{SO}_4$  or 9.07 Gm per cent  $\text{KHSO}_4$
- (3) micro carbonate-tartaric acid reagent
  - (a) 40 Gm of anhydrous sodium carbonate c.p. are dissolved in 400 ml of water
  - (b) 5 Gm of crystalline copper sulfate c.p. and 7.5 Gm. of tartaric acid are dissolved in 150 ml of distilled water. Solution (b) is poured into solution (a) with constant stirring
  - (c) 0.7134 Gm of potassium iodate, 10 Gm of potassium iodide and 18.4 Gm of potassium oxalate are dissolved in 250 ml of distilled water and added to the mixture of (a) and (b) in a 1000 ml volumetric flask. After cooling the volume is made up to 1000 ml with distilled water
- (4) N/200 sodium thiosulfate solution
- (5) 0.25 per cent starch solution
- (6) N/1  $\text{H}_2\text{SO}_4$  or 5 N  $\text{H}_2\text{SO}_4$

*Procedure*

Five ml of Folin filtrate representing 0.5 ml of blood (see non-protein nitrogen determination p. 142) and 5 ml of tartaric acid reagent are placed into a large test tube. Each tube is covered with

a small inverted beaker and immersed in a boiling water bath for fifteen minutes. The tubes are cooled under running cold water and the contents acidified with 1 ml of 5 N  $H_2SO_4$  or 5 ml of N/1  $H_2SO_4$ . The liberated iodine is titrated with N/200 sodium thiosulfate, using a few drops of starch indicator towards the end of the titration. The end-point is sharp and permanent.

The blank titration is determined after heating the reagents with water instead of blood filtrate. The blank titration of 5 ml of reagent should require about 20 ml of N/200 thiosulfate and is constant. The reagent (carbonate-tartaric acid) may be used to standardize the thiosulfate.

### *Calculation*

The difference between titer of the blank and of the sample multiplied by the copper-factor of the thiosulfate used, gives the copper reduced. One ml. of N/200 thiosulfate equals 0.318 mg copper. Table 16 should be consulted for the conversion of copper into terms of glucose, the table gives also data from which the difference in titration in ml N/200 thiosulfate for 5 ml of reagent plus 5 ml of blood filtrate may be converted directly into percentage of sugar in blood.

The normal fasting values of blood sugar vary between 70 and 120 mg per cent.

High fasting blood sugar levels are found in

- (1) diabetes mellitus
- (2) hunger hyperglycemia
- (3) hyperthyroidism
- (4) increased adrenal function, or after adrenalin injections
- (5) after ether-chloroform anesthesia (in any case of anesthesia)
- (6) asphyxia
- (7) often during periods of fever
- (8) in some forms of acromegalia
- (9) convulsions (epilepsy, eclampsia)

Decreased fasting blood sugar levels are found in

- (1) hyperinsulinism (adenoma, hyperplasia of islets of Langerhans) after insulin injections and in adrenal insufficiency
- (2) hypothyroidism
- (3) acute yellow atrophy of the liver

SUGAR VALUES IN SPINAL FLUID<sup>3</sup>

normal fasting adult	40- 70 mg per cent sugar
normal fasting child (to 10 years)	70- 90 mg per cent sugar
functional mental disease	70- 95 mg per cent sugar
lesions of central nervous system	30-110 mg per cent sugar
epidemic encephalitis	70-110 mg per cent sugar
suppurative meningitis	0- 25 mg per cent sugar
tuberculous meningitis	18- 36 mg per cent sugar
brain abscess	70-110 mg per cent sugar
brain tumor	70-100 mg per cent sugar

## CARBOHYDRATE TOLERANCE TESTS

The presence of a normal or almost normal fasting blood sugar level does not prove that the carbohydrate metabolism of the organism is intact. Therefore several methods have been devised to test the tolerance of the organism for carbohydrates. A few methods are described below

*One Hour, Two-Dose Sugar Tolerance Test (Eaton and Ross)\**

- (1) Fasting blood and urine samples, No 1 are collected
- (2) Patient receives by mouth first dose of glucose (50 Gm. dissolved in 325 ml of water). From one to 2 minutes are allowed for its ingestion
- (3) Thirty minutes after ingestion of glucose, blood and urine samples, No 2 are collected
- (4) Patient receives second dose of glucose same as above
- (5) Thirty minutes after ingestion of glucose blood and urine samples No 3 are collected.
- (6) Blood and urine examinations are performed in the usual manner

*Notes*

- (1) A normal tolerance gives a normal fasting No 1 blood sugar and a negative No 1 urine sugar. Blood No 2 shows a rise not exceeding 75 mg and a negative No 2 urine sugar. Blood No 3 is less,

\* According to J. Kolmer Approved Laboratory Technique, Appleton-Century Company London New York 3rd edition p 735

the same or does not exceed the sugar content of No 2 by more than 5 mg and a negative No 3 urine sugar

TABLE 17 — *Decreased Glucose Tolerance*

Condition		Time after glucose				
		Fasting	30 min.	60 min.	90 min.	120 min.
Normal	Venous sugar	90	125	130	100	85
	Arterial sugar	90	135	155	105	90
	A V Difference	0	+10	+25	+5	+5
	Serum P	4 0	3 4	2 9	2 5	2 8
	R Q	0 82	0 84	0 88	0 90	0 95
Decreased hepatic glycogenesis	Venous sugar	60	140	170	130	90
	Arterial sugar	60	150	190	145	100
	A V Difference	0	+10	+20	+15	+10
	Serum P	4 0	3 6	3 0	2 8	3 1
	R Q	0 82	0 84	0 86	0 89	0 92
Increased hepatic glycogenesis	Venous sugar	110	165	190	170	135
	Arterial sugar	110	180	210	185	140
	A. V Difference	0	+15	+20	+15	+5
	Serum P	4 0	3 5	3 1	2 6	2 6
	R Q	0 86	0 88	0 90	0 95	0 96
Diabetes mellitus	Venous sugar	180	200	310	340	350
	Arterial sugar	180	265	318	350	355
	A V Difference	0	+5	+8	+10	+5
	Serum P	4 0	3 9	4 0	4 0	4 0
	R Q	0 76	0 76	0 77	0 78	0 77
Hyperpituitarism	Venous sugar	110	150	170	160	130
	Arterial sugar	110	155	180	165	135
	A. V Difference	0	+5	+10	+5	+5
	Serum P	4 0	3 8	3 8	3 9	4 0
	R. Q	0 79	0 80	0 81	0 82	0 82

(2) In diabetes the blood sugar of No 3 is 10 or more mg. higher than No 2 the urines may or may not be positive for sugar according to the severity of the disease

(3) Should the No 3 blood sugar rise sufficiently to classify the tolerance as being abnormal and all the urines are negative, then a



fourth sample of urine may be collected one hour after No 3 which in most cases will be positive for sugar

*Tolerance Test with Oral Administration of 100 Gm of Glucose<sup>10</sup>*

In a normal fasting person (12-16 hours after the last meal) the blood sugar level is doubled within thirty minutes after intake of 100 Gm of glucose in tea. The blood sugar generally returns to the starting level  $1\frac{1}{2}$ -3 hours later, often levels lower than the starting one are reached. In a diabetic person the rise is more marked and

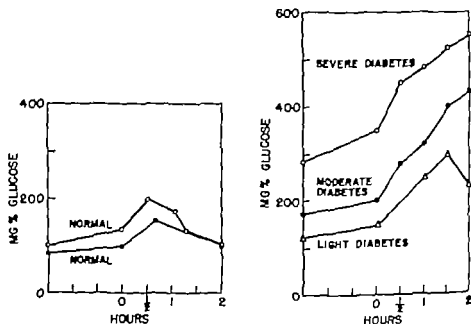


FIG 43 Tolerance tests with oral administration of 100 grams of glucose

the hyperglycemic period lasts longer (see table 17 and fig 43). A higher rise of the blood sugar curve may also be caused by hunger, prolonged period of time after last meal, and by a prolonged carbohydrate-free diet.

*Tolerance Test according to Staub-Traugott with Modification of L. Pollak, c.s.<sup>11-14</sup>*

The fasting patient receives 2 doses of 50 Gm. of glucose each orally, the time interval being  $1\frac{1}{2}$  hours. The resulting blood sugar curve is characterized by the fact that at ninety minutes after administration of the first dose the blood sugar level is only 30 mg per

cent higher than the fasting level. Since it has been found by experience that the ninety minute value is the most significant one sugar determinations between zero and ninety minutes may be omitted in this method (first part of double load). During the second part of the test 10 after the second dose of glucose blood sugar determinations are performed at 30, 60 and 120 minutes (time counted from the time of second administration of glucose). Normally the sugar level will rise very slightly or not at all after the second dose in any case the fasting level will be reached again after two hours. Under pathologic conditions the hyperglycemic reaction during the first period is prolonged, i.e. at ninety minutes a more or less pronounced rise is noted. During the second part the level may continue

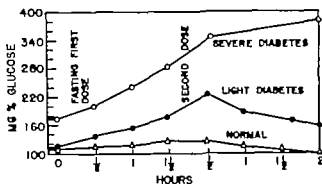


FIG. 44 Tolerance test according to Staub-Traugott

to rise nevertheless it may return to the fasting level after two hours, counted from the point of administration of the second dose of glucose. In other cases a delayed return to normal has been found to accompany the abnormally high rise during the second part. This latter behavior is characteristic for an insufficiency of the islet cells. (See curves, fig. 44.)

#### *Tolerance Test by Dosage with White Bread according to C. v. Noorden<sup>18</sup>*

After fasting for 12-16 hours the patient receives 1, 2, 3 and 4 white bread units in one hour intervals (one white bread unit equals 25 Gm. of white bread). Blood sugar analyses are carried out before each bread intake as well as one and 2 hours after the last intake. Fig. 45 shows the resulting curves.

Certain pathologic conditions other than diabetes mellitus can

cause a decreased carbohydrate tolerance. Other conditions again may cause an increase of the tolerance.

### *Decreased carbohydrate tolerance*

The decreased tolerance may be caused by 3 factors

(1) decreased ability of the liver to form glycogen. This occurs in all acute rapidly progressing liver diseases, in acute and subacute yellow atrophy of the liver, in phosphorus-chloroform and carbon tetrachloride poisoning, in acute alcohol poisoning, in toxic hepatitis, in hepatitis with disease of the gall bladder in congestive jaundice and catarrhal jaundice (see table 17) <sup>18</sup>

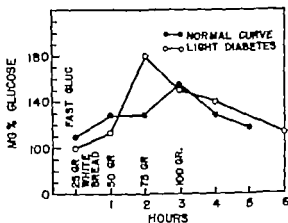


FIG. 46 Administration of white bread according to C. van Noorden

(2) glycogen deficiency of the liver. This group includes hyperthyroidism, hyperactivity of the adrenals, acidosis, chronic malnutrition, cachexia. Also all acute occurring infectious diseases, such as lobar pneumonia, diphtheria, scarlet fever (table 17).

(3) decreased utilization of sugar in the tissues. The most important clinical manifestation is in diabetes mellitus (table 17) and hyperpituitarism.

### *Increased carbohydrate tolerance*

The alimentary rise of blood sugar after administration of carbohydrates is less than in the normal person. From the practical clinical point of view these cases may be divided into 2 groups.

(1) decreased glycogenolysis in the liver. In this group belongs

hypothyroidism (myxedema, cretinism), Addison's disease, diabetes mellitus essential hypertension, late stage of pregnancy, severe anemia

(2) Increased utilization of sugar in the tissues a typical representative of this group is hyperinsulinism functional disturbances accompanying hyperplasia, adenoma and carcinoma of the islands of Langerhans in the pancreas (table 18) excessive ingestion of carbohydrates also belongs to this group

TABLE 18—*Increased Glucose Tolerance*

Condition		Time after glucose				
		Fasting	30 min.	60 min.	90 min.	120 min.
Decreased hepatic glycogenolysis	Venous sugar	65	70	80	74	68
	Arterial Sugar	65	73	90	80	75
	A V Difference	0	+3	+10	+6	+7
	Serum P	4.0	3.2	2.6	2.2	2.2
	R. Q	0.82	0.85	0.80	0.94	0.96
Hyperinsulinism	Venous sugar	50	55	58	54	48
	Arterial sugar	50	70	90	65	58
	A V Difference	0	+15	+32	+11	+10
	Serum P	3.8	3.0	2.2	1.0	1.4
	R. Q	0.85	0.90	0.94	0.96	0.96
Hypopituitarism	Venous sugar	70	78	82	76	70
	Arterial sugar	70	90	120	110	95
	A V Difference	0	+12	+38	+34	+25
	Serum P	4.0	3.2	2.6	2.4	2.4
	R. Q	0.82	0.80	0.90	0.95	0.96

(3) hypopituitarism (table 18)

With all these tests it can be demonstrated that the sugar content of venous blood differs from that of arterial (capillary) blood. The administration of carbohydrates also causes a change of inorganic serum phosphorus level. In the normal person the phosphorus content will drop 1-1.5 mg. per cent (maximum reached after 30 minutes, return to normal after 4-5 hours).

Tables 17 and 18 show blood sugar levels (arterial and venous) and inorganic phosphorus levels under various conditions.

DETERMINATION OF FRUCTOSE<sup>17</sup> (LEVULOSE)

*Principle of the method* In contrary to glucose, fructose is able to reduce phosphomolybdic acid to a blue compound (according to Folin) which can be titrated with N/100 potassium permanganate in the cold till colorless. The reduction of phospho-molybdic acid is proportional to the fructose concentration in the sample, but not specific, since dioxyacetone, methyl-glyoxal, and glycerine-aldehyde show the same property. However, these compounds do not ordinarily occur in appreciable amounts in normal blood and therefore they do not interfere with the fructose determination in experimental fructosemia. Glucose causes only a minimal reduction accounted for in the blank run. When blood-glucose is to be determined together with fructose, the fructose is determined first, and then the total reduction is estimated according to Hagedorn-Jensen. From this latter value the reduction value for fructose is subtracted. For clinical purposes it suffices to put the reduction of 100 mg. of fructose as equal to 96 mg. of glucose.

The estimation of fructose is performed in the protein free filtrate, prepared either according to Folin Wu or Hagedorn-Jensen with the modification as indicated by Steinitz and Riessen.

*Reagents*

(1) (a) For deproteinizing according to Folin Wu see uric acid, p. 195

(b) for deproteinizing according to Hagedorn-Jensen with the modification of Steinitz and Riessen<sup>18</sup>  $\alpha$ ) 0.45 per cent zinc sulfate solution.  $\beta$ ) sodium hydroxide in a 100 ml volumetric flask 12.5 ml of N/1 NaOH are made up to the mark with distilled water.

(2) molybdate reagent (a) 40 g of sodium molybdate are dissolved in 100 ml of distilled water. (b) 50 ml of 85 per cent phosphoric acid 40 ml of 20 volumes per cent sulfuric acid and 20 ml of glacial acetic acid are mixed with constant stirring. Before use equal parts of solutions (a) and (b) are mixed. This mixture will keep for 2-3 days only when stored in the refrigerator. Solutions (a) and (b), when stored separately, will keep indefinitely.

(3) N/100 potassium permanganate solution

(4) ferrous phenanthroline indicator (red-ox indicator is deep red as ferrous salt, turns colorless (faintly blue) upon oxydation to the ferric salt).

*Preparation of the indicator*

To prepare phenanthroline<sup>19</sup> 20 Gm of ortho-phenylene-diamine 110 Gm of glycerin 52.5 Gm of arsenic acid and 100 ml of concentrated  $H_2SO_4$  are mixed. The reaction is started by careful heating with frequent shaking. When it has subsided it is heated to a slow boil for 2-3 hours and after that time it is diluted with at least twice its volume of water. After filtration NaOH is added until the reaction is weakly acid causing precipitation of a resinous mass, which forms a solid lump after shaking and cooling. The supernatant is decanted filtered by suction and the gummy residue which contains a considerable amount of phenanthroline is thoroughly washed with hot water to which a small amount of  $H_2SO_4$  has been added. Filtrate and washings are pooled and the  $HgCl_2$ -double salt of phenanthroline is precipitated with concentrated sublimate solution. The precipitate is separated by suction through a Buechner funnel and after suspension in water it is decomposed by  $H_2S$  in the heat. After removal of  $HgS$  by suction the filtrate is concentrated and NaOH is added until a weak alkaline reaction is observed. Now it is evaporated to dryness heated to approximately  $140^\circ C$  for several hours and the residue is extracted with methyl alcohol. The extract is again evaporated to dryness and the residue is distilled in vacuo in a retort (sausage flask) ( $230-240^\circ C$  and 11 mm Hg). Finally the compound is recrystallized from a large volume of water. Yield up to 12 gm (melting point of the monohydrate  $105^\circ C$ ). Ten grams of phenanthroline and 2.0 Gm of ferrous sulfate are dissolved in a small amount of water and the solution is saturated with KBr. The crystals are separated by suction and washed with a very small amount of ice water.

A freshly prepared solution of 20 mg of these crystals (ferrous phenanthroline bromide) in 25 ml of water is used as indicator.

*Procedure*

(a) Determination of fructose with deproteinizing according to Folin-Wu

In a test tube 2 ml of molybdate mixture (2) are added to 2 ml. of Folin Wu filtrate and heated in a boiling water bath for exactly 25 minutes. After cooling the titration is performed with N/100 potassium permanganate (freshly prepared each time) 0.5 ml. of indicator (4) having been added. The original blue color gradually

changes to red and the first drop of excess permanganate brings about a sharp change from red to colorless. As the blue molybdenum reaction product is stable, the titration may also be performed on the following day. If more than 2 ml. of permanganate is required, less filtrate must be used. In this method it is important to use equal volumes of molybdate mixture and filtrate.

### Calculation

$$\frac{(\text{ml. N/100 KMnO}_4 - 0.06) \times 500}{2.70} = \text{mg. per cent fructose in blood.}$$

0.06 ml. correspond to the blank value of blood glucose of approximately 100 mg. per cent (this value increases by 0.023 ml. of N/100 KMnO<sub>4</sub> for each 100 mg. of blood glucose). 2.70 is the specific reduction for fructose and 500 is the factor for 100 ml. of blood (dilution factor, 2 ml. of filtrate corresponding to 0.2 ml. of blood).

(b) Determination of fructose with deproteinizing according to Hagedorn-Jensen

Two tenth ml. (0.2 ml.) of whole blood is blown into a test tube containing 2 ml. of 0.45 per cent ZnSO<sub>4</sub> solution and 0.8 ml. of NaOH and the pipet is rinsed several times with the deproteinizing mixture. After heating for three minutes in a boiling water bath it is filtered through a dry filter. One and five-tenths of a milliliter of filtrate (corresponding to 0.1 ml. of blood) is mixed with 1.5 ml. of phosphomolybdic acid reagent (2) and heated in a boiling water bath for 20 minutes.

### Calculation

$$\frac{(\text{ml. N/100 KMnO}_4 - 0.05) \times 1000}{2.70} = \text{mg. per cent fructose.}$$

### Example for (a)

Permanganate used up 0.35 ml. According to the Hagedorn-Jensen method the reduction corresponds to a glucose content (glucose + fructose) of 145 mg. per cent.

From the permanganate titration the fructose content is calculated according to the above mentioned formula

$$\frac{(0.35 - 0.06) \times 500}{2.70} = \frac{0.29 \times 500}{2.70} = \frac{145}{2.7} = 53.7 \text{ mg. per cent}$$

$$\text{glucose content} = 145 - \frac{53.7}{0.96} = 145 - 55.9 = 89.1 \text{ mg. per cent}$$

The calculation for (b) is the same as above

The fructose tolerance test is often used clinically as a liver function test. If the island apparatus is intact, the blood sugar level is less influenced by fructose administration than by the administration of glucose because the liver takes up the resorbed fructose immediately and utilizes it far more easily than glucose for the glycogen synthesis. Originally the liver function test with fructose was limited to the test for fructose excretion in urine. The more refined methods are based upon administering to the fasting patient (12-16 hours of fasting) 40-50 Gm. of dextrose-free fructose dissolved in 200 ml of water or tea. Blood for analysis is taken every thirty minutes over a period of two hours.

The normal curve is characterized by a maximum of 30 mg per cent above the starting value after 30 minutes and by a return to normal after 1-1½ hours. In cases of decreased ability of the liver to convert fructose into glycogen the fructose content of blood increases by more than 35 mg per cent (up to 135 mg per cent) and does not return to normal after two hours. This phenomenon is found less in chronic than in acute liver diseases (acute yellow atrophy, chloroform phosphorus-arsenic poisoning, acute catarrhal jaundice). A decreased fructose tolerance is also encountered in hyperthyroidism (Basedow's disease).

#### DETERMINATION OF GALACTOSE

*Principle of the method* After yeast fermentation of the blood glucose the yeast-resistant galactose is determined according to Hagedorn Jensen.

##### *Reagents*

(1) yeast suspension 2 Gm. of ordinary fresh baker's yeast is suspended in 20 ml of 0.85 per cent NaCl solution. The suspension is centrifuged at high speed, the supernatant liquid is decanted and the sediment washed several times with 20 ml portions of 0.85 per cent NaCl until a clear supernatant results. This is usually the case after 4-6 centrifugations and washings. Now the sediment is taken up in 20 ml of 0.85 per cent NaCl solution and placed in a water bath at



30°C for thirty minutes. After this time the yeast is again centrifuged, the supernatant is decanted and the residue is again taken up in 20 ml of 0.85 per cent NaCl solution and centrifuged. This procedure is repeated twice more. Finally the sediment is taken up in 15 ml of 0.85 per cent NaCl solution. This yeast suspension must be tested before each analysis for the ability to ferment glucose and leave galactose intact.

(2) In some cases it is impossible to find suitable yeast then the yeast (preferably the strain S. Ludwigii) must be grown in clarified beer wort as follows

(a) Before use equal parts of beer wort and water are mixed, and one tea spoon of egg albumin per liter solution is added. This mixture is heated for 8 hours in a steam pot, filtered and filled into flasks or tubes. If a precipitate forms after some time, the beer wort must be refiltered and sterilized. The tubes are inoculated with the stock culture incubated for 24 hours at 28°C and stored in the refrigerator, under these conditions they will keep over a period of time. The amount of yeast required for the analysis is obtained by inoculation of 200 ml of beer wort with the entire content of a stock culture tube and incubating at 28°C for twenty four hours. The yeast forms a large amount of sediment which is washed 5 times with m/15-m/25 phosphate solution. Finally a 1 per cent suspension in this buffer mixture is prepared.

(b) phosphate solution 95 parts of primary (m/15) and 5 parts of secondary (m/15) phosphate are mixed (see appendix p 384), 0.3 Gm of sodium oxalate is dissolved in 100 ml of this mixture. Oxalate is added to the buffer to prevent coagulation of the blood. All other reagents see determination of blood sugar p 242

### *Procedure*

The required number of test tubes (plus 3 tubes for blank runs) is filled with 0.5 ml. of water. With an exactly calibrated capillary pipet 0.1 ml. of blood is drawn from the finger tip and after the pipet has been wiped off carefully on the outside the contents are blown into the tube. The pipet is rinsed by drawing up and blowing out the mixture in the tube three times. One half (0.5) ml. of yeast suspension is added to all tubes and blank tubes and samples are placed into an incubator at 30 C for approximately one hour and shaken every fifteen minutes.

After incubation 1 ml. of N/10 NaOH and 5 ml. of 0.45 per cent zinc sulfate is added to each tube. The tubes containing the samples are immersed in a water bath of 80°C for a few minutes (2-3) and filtered while hot into Hagedorn-Jensen tubes through a small plug of cotton, which has been washed with hot water (fig. 41). The tubes are rinsed 3 times with 3 ml. of hot water each time and the wash water is also filtered. The blanks are filtered cold. (The tubes must not be heated, but must be washed 3 times with hot water.) Heating of the blanks has to be avoided because the filtrate may become cloudy. The rest of the procedure is the same as described for the determination of blood sugar according to Hagedorn-Jensen.

### Calculation

#### (a) nonspecific reduction.

After fermentation of the glucose by yeast each blood filtrate still contains a number of compounds which are able to reduce potassium ferrioyanide in the heat, such as uric acid, creatin creatinine, etc. This reduction is called nonspecific reduction and can be expressed in mg. per cent glucose according to the Hagedorn-Jensen table.

The amount of nonspecific reduction varies with different individuals but is relatively constant in each person and represents glucose value of the total amount after fermentation.

(b) The value for galactose is calculated according to the following formula: mg. per cent galactose =  $(C - B) \times 1.25$

C = mg. per cent sugar after galactose administration and yeast fermentation

B = mg. per cent sugar after yeast fermentation of the fasting blood (non-specific reduction). The factor 1.25 results from the fact that the reducing power of 100 mg. galactose equals that of 80 mg. of glucose, ( $\frac{100}{80} = 1.25$ )

(c) glucose content and that of galactose is calculated as follows:  
mg. per cent glucose =  $D - [A + (C - B)]$  or  
=  $D - A - C + B$

D = mg. per cent sugar after galactose administration without yeast fermentation.

A = mg. per cent sugar of the blank run.

B = mg. per cent sugar after yeast fermentation without galactose administration. Nonspecific reduction.

C = mg per cent sugar after yeast fermentation and galactose administration

*Example*

thiosulfate used up by the blank (re agents)	1.97 ml (5 mg per cent glucose) (A)
thiosulfate used up by blood after yeast ferment before galactose intake	1.84 ml (27 mg per cent glucose) (B)
thiosulfate used up by blood after yeast ferment and galactose intake	1.44 ml (99 mg per cent glucose) (C)
thiosulfate used up by blood after galactose intake without yeast ferment (total reduction)	0.84 ml (206 mg per cent glucose) (D)

From this can be calculated

(1) galactose content  $(C - B) \times 1.25 = (99 - 27) \times 1.25 = 90$  mg per cent galactose

(2) glucose content including nonspecific reduction

$D - A - C + B = 206 - 5 - 99 + 27 = 129$  mg per cent glucose

The normal tolerance dose for galactose is 30-40 Gm. Galactose is taken up mainly by the liver, like fructose, and converted into glycogen but the endocrine glands have a greater influence upon the galactose tolerance than upon the glucose tolerance. A decreased tolerance is found in impaired liver functions (galactose test according to Bauer,<sup>20, 21</sup> during menstruation and pregnancy, in hyperpituitarism, less often in hyperthyroidism. It is also encountered in disfunction of the sex glands. An increased tolerance is found in hypopituitarism and hypothyroidism.

#### GALACTOSE TOLERANCE TEST<sup>22</sup>

The patient is starved over night no breakfast or morning tea being allowed. A dose of 40 Gm. of galactose, dissolved in 250 ml.

of water, is given by mouth. It is necessary to use hot water and cool subsequently to obtain complete solution. Blood is taken before and  $\frac{1}{2}$ , 1,  $1\frac{1}{2}$  and 2 hours after the solution has been drunk.

Fig 46 shows a typical normal and a typical abnormal curve. The maximum blood galactose may occur at  $\frac{1}{2}$ , 1 or  $1\frac{1}{2}$  hours (usually 1 hour) and the sum of these four values, for which the term "galactose index" (G I) is suggested, appears to be the best criterion for purposes of comparison and tends to be about double the highest value.

There is usually a fairly close correlation between the galactose index and the highest value and it is evident that a peak value of 80

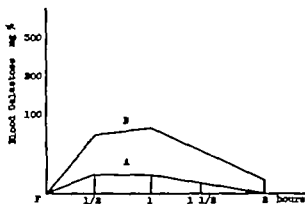


FIG. 46 GALACTOSE TOLERANCE CURVES

mg of galactose per 100 ml or a G I of about 100 may be taken as the upper normal limit. The average normal G.I. is 68.

Normal figures were recorded in diabetes and in obstructive jaundice, and impairment of function was demonstrated in toxic jaundice and in hyperthyroidism.

### DIOXYACETONE<sup>22</sup>

*Principle of the method* Dioxyacetone  $\text{HOH}_2\text{C}-\overset{\text{O}}{\underset{|}{\text{C}}}-\text{CH}_2\text{OH}$  is able to reduce potassium ferricyanide in the cold. This reaction is quantitative and stoichiometric. The excess potassium ferricyanide is determined iodometrically. Other compounds present in blood

and also capable of reducing in the cold are taken care of by a blank run

Since dioxyacetone has never yet been proven to be an intermediary metabolic product, its determination is only performed in cases of experimental dioxyacetoneemia

### *Reagents*

Same as described for the determination of blood sugar according to Hagedorn-Jensen, (p 242)

### *Procedure*

The required number of test tubes plus 3 tubes for blank runs is filled with 5 ml of 0.45 per cent zinc sulfate solution and 1 ml. of N/10 NaOH solution each. A colloidal solution of zinc hydroxide results. With an exactly calibrated pipet 0.1 ml of blood is drawn from the finger tip and after the outside of the pipet has been wiped clean, the blood is blown into the colloidal zinc-hydroxide solution. The pipet is rinsed by drawing up and blowing out the deproteinizing mixture 3 times. The tubes containing the sample as well as the tubes for the blank (zinc hydroxide without blood) are placed into a boiling water bath for one and one-half minutes. After cooling the samples and the blanks are filtered through cotton which has been washed with boiling water, into Hagedorn-Jensen tubes (fig 41). The tubes are rinsed 3 times with 3 ml of boiling water, and the wash water is also filtered. After the addition of 2 ml of potassium ferrocyanide (3) (exactly measured) the tubes are allowed to stand at room temperature for 16-24 hours. Then 2 ml of the acid zinc sulfate-potassium iodide mixture (4) and a few drops of starch are added to each tube and the liberated iodine is titrated from a micro-burette with N/200 thiosulfate till colorless.

When blood sugar is to be determined together with dioxyacetone (heat reduction) \* all tubes must be left standing at room temperature for 16-24 hours after the addition of potassium ferrocyanide only then the tubes marked for reduction in the heat are immersed in a boiling water bath for fifteen minutes and titrated. When these tubes are heated without digestion at room temperature the results are too low

\* In this case twice the number of tubes must be set up

*Calculation*

Cold reduction of the reagents subtracted from the cold reduction of the sample multiplied by 0.1 corresponds to the dioxycetone content of the sample (but if dioxycetone is analyzed in pure solutions 1 ml. of N/200 sodium thiosulfate corresponds to 0.09 mg. of dioxycetone. By deproteinization of the blood the dioxycetone loses about 10 per cent of its reduction power)

*Example*

cold reduction of reagents	1.08 ml (A)
hot reduction of reagents	1.07 ml (B)
cold reduction of blood before dioxycetone load	1.88 ml (C)
cold reduction of blood after dioxycetone load	1.65 ml (D)
hot reduction of blood before dioxycetone load	1.34 ml (E)
hot reduction of blood after dioxycetone load	1.15 ml (F)

From these figures the following values are calculated

(1) dioxycetone content of blood in mg. per cent =  $(C - D) \times 0.1 \times 1000 = (1.88 - 1.65) \times 100 = 0.23 \times 100 = 23$  mg. per cent.

(2) glucose content before load (see table 12). According to the table a thiosulfate requirement of 1.34 ml. = 117 mg. per cent with a blank value of 1.07 ml. = 5 mg. per cent corresponds to glucose content of 112 mg. per cent.

(3) glucose content after load (together with dioxycetone). In order to calculate the glucose content after administration of dioxycetone the following formula must be used

$$2.00 - [(B - F) - (C - D)] = 2.00 - [(1.07 - 1.15) - (1.88 - 1.65)] = 1.41$$

1.41 ml. of thiosulfate read from the Hagedorn-Jensen table correspond to a glucose content of blood of 100 mg. per cent.

LIVER FUNCTION TEST WITH DIOXYACETONE<sup>21</sup>

The fasting patient receives 40 Gm. of dioxycetone dissolved in 2 cups of tea. Blood is drawn for blood sugar and dioxycetone

determinations before administration of dioxycetone, and five, fifteen, thirty and sixty minutes afterwards. Normally the average value will not exceed 8 mg per cent of dioxycetone. In cases of liver damage higher values up to 90 mg per cent within 5-15 minutes are found.

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## Chapter VIII

# Ketobodies and Polyvalent Alcohols

### QUALITATIVE TEST FOR KETONEMIA IN SMALL AMOUNTS OF SERUM<sup>1</sup>

*Principle of the method* Acetone in the presence of nitroprusside sodium and ammonia develops a violet (permanganate-like) color (Rothera's test). This test is simplified by using dry powdered reagents.

#### *Reagent*

1 Gm. of nitroprusside sodium and 23 Gm. of ammonium sulfate are finely ground and thoroughly mixed in a mortar. Now 20 Gm. of anhydrous sodium carbonate are added and mixed completely in a well-closed jar.

#### *Procedure*

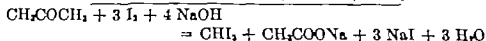
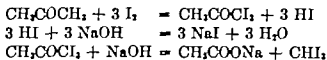
A small pinch of the powder is placed on a spot plate according to Feigl<sup>2</sup> and one drop of serum\* is added and mixed with a glass rod. The presence of acetone is indicated by the appearance of a violet color.

The minimal blood level of acetone bodies giving a definitively positive test is approximately 10 mg. per cent.

### SEMIMICRO-DETERMINATION OF ACETONE AND ACETOACETIC ACID

*Principle of the method* Acetoacetic acid and preformed acetone are distilled from the deproteinized blood filtrate and absorbed in a measured amount of iodine solution of known titer. The excess iodine is titrated with thio-sulfate.

#### *Reaction equation*



The same test may be used for urine analysis.



*Reagents*

- (1) 10 per cent sodium tungstate solution
- (2)  $\frac{1}{2}$  N  $\text{H}_2\text{SO}_4$  or 9.076 Gm per cent  $\text{LiHSO}_4$
- (3) 20 per cent  $\text{NaOH}$ , prepared from  $\text{NaOH}$  c p
- (4) 20 per cent  $\text{H}_2\text{SO}_4$
- (5) N/200 iodine solution, prepared freshly each time from N, solution
- (6) N/200 sodium thiosulfate solution
- (7) 0.25 per cent starch solution

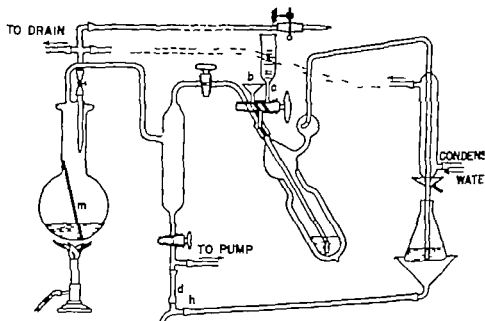


FIG 47 DISTILLING APPARATUS FOR IRON AND ACETONE.

*Procedure*

According to Folin Wu (see Uric Acid p 195) 1 ml of oxala blood (plasma or serum) is deproteinized and filtered. Five ml filtrate, corresponding to 0.5 ml of blood and 2 ml of sulfuric acid are transferred to the distilling apparatus (fig 47). Into the receiver are placed 3 ml of N/200 iodine solution to which  $\text{NaOH}$  is added until the brown color disappears (using a dropping bottle). During the first five minutes of the distillation the receiver flask is placed with the condenser tube reaching below the surface of the alkaline iodine solution. Then the receiver is lowered and the distillation

continued for five more minutes. After the distillation is completed the receiver flask is removed and 20 per cent sulfuric acid is added with cooling until a brown color due to liberated iodine appears. After the addition of several drops of starch solution the titration is performed from a micro-burette with N/200 sodium thiosulfate till colorless.

The accuracy of the method depends on several factors

(1) The analytical data are correct only if a blank run does not use up any alkaline iodine solution in the receiver (The amount of thiosulfate required by 3 ml of iodine solution before distillation must be identical with the amount required after distillation of the reagents without blood) This can only be achieved when the entire apparatus is constructed in one piece Rubber connections are an unending source of errors.

(2) No alcohol, acetone or ether may be used in the room where the distillation is carried out

(3) All glassware used must be soaked in cleaning solution over night rinsed with water, and dried in an oven (beware of organic solvents)

### Calculation

As seen from the reaction equation 1 molecule acetone corresponds to 6 iodine atoms. Consequently 1 ml of N/200 thiosulfate solution corresponds to  $\frac{58}{6 \times 200 \times 1000} = 0.0184$  mg of acetone As 5 ml. of filtrate = 0.5 ml of blood have been used for the analysis, the blank value minus value for the sample multiplied by 0.6 results in the acetone content expressed in mg per cent (table 19)

### Example

N/200 thiosulfate used up by the blank	2.86 ml
N/200 thiosulfate used up by the sample	2.11 ml
difference	0.75 ml
0.75 ml $\times$ 0.6 = acetone content of 7.2 mg per cent.	

### MICRO-DETERMINATION OF ACETONE AND ACETOACETIC ACID<sup>3</sup>

The following method, which is based on the method of M. Ljungdahl<sup>4</sup> allows the determination of acetone bodies accurately and

TABLE 19—Table for the calculation of acetone content

Ml N/200  $\text{Na}_2\text{S}_2\text{O}_3$  =  $\gamma$  acetone

	0	1	2	3	4	5	6	7	8	9
0 0		0 48	0 96	1 45	1 93	2 42	2 90	3 38	3 87	4 35
0 1	4 84	5 32	5 81	6 29	6 78	7 26	7 74	8 23	8 71	9 20
0 2	9 68	10 16	10 65	11 13	11 61	12 10	12 58	13 07	13 55	14 04
0 3	14 52	15 00	15 49	15 97	16 46	16 94	17 42	17 91	18 39	18 88
0 4	19 36	19 84	20 32	20 81	21 29	21 78	22 26	22 75	23 23	23 72
0 5	24 20	24 68	25 17	25 65	26 13	26 62	27 10	27 59	28 07	28 56
0 6	29 04	29 52	30 00	30 49	30 97	31 46	31 94	32 43	32 91	33 40
0 7	33 88	34 36	34 85	35 33	35 82	36 30	36 78	37 27	37 75	38 24
0 8	38 72	39 20	39 69	40 17	40 66	41 14	41 62	42 11	42 59	43 08
0 9	44 01	44 53	45 01	45 50	45 98	46 46	46 95	47 43	47 92	48 40
1 0	49 88	49 37	49 85	50 34	50 82	51 30	51 79	52 27	52 76	53 24
1 1	53 72	54 21	54 69	55 18	55 66	56 14	56 63	57 11	57 60	58 08
1 2	58 56	59 05	59 53	60 01	60 50	60 98	61 47	61 95	62 44	62 92
1 3	63 40	63 89	64 37	64 86	65 34	65 82	66 31	66 79	67 28	67 76
1 4	68 24	68 73	69 21	69 70	70 18	70 66	71 15	71 63	72 12	72 60
1 5	73 08	73 57	74 05	74 54	75 02	75 50	75 99	76 47	76 96	77 44
1 6	77 92	78 41	78 89	79 38	79 86	80 34	80 83	81 31	81 79	82 28
1 7	82 76	83 25	83 73	84 22	84 70	85 18	85 67	86 15	86 64	87 12
1 8	87 60	88 09	88 57	89 06	89 54	90 02	90 51	90 99	91 48	91 96
1 9	92 44	92 93	93 41	93 89	94 38	94 86	95 35	95 83	96 32	96 80
2 0	97 28	97 77	98 25	98 74	99 22	99 70	100 19	100 67	101 16	101 64
2 1	102 12	102 61	103 09	103 57	104 06	104 54	105 03	105 51	105 99	106 48
2 2	106 96	107 45	107 93	108 42	108 90	109 38	109 87	110 35	110 84	111 32
2 3	111 80	112 28	112 77	113 26	113 74	114 22	114 71	115 19	115 67	116 16
2 4	116 64	117 13	117 61	118 10	118 58	119 06	119 55	120 03	120 52	121 00
2 5	121 48	121 97	122 45	122 94	123 42	123 90	124 39	124 87	125 36	125 84
2 6	126 32	126 80	127 29	127 78	128 26	128 74	129 23	129 71	130 20	130 68
2 7	131 16	131 65	132 13	132 62	133 10	133 58	134 07	134 55	135 04	135 52
2 8	136 00	136 49	136 97	137 46	137 94	138 42	138 91	139 39	139 88	140 36
2 9	140 84	141 33	141 81	142 29	142 78	143 26	143 75	144 23	144 72	145 20
3 0	145 68									

quickly in 0.2 ml. of blood or serum by means of a simple and easily handled apparatus. The distillation apparatus (fig. 48) consists of three parts:

(1) The distilling flask (A) which is a 50 ml. Erlenmeyer flask connected to the distilling headpiece by a ground joint.

(2) The distilling headpiece. When in use the connection is secured by a pair of elastic springs (B). The headpiece consists of a glass tube



distilling top piece. This tube (C) is extended to a capillary (D), running parallel to the axis. During the distillation, the tube is closed by a cork stopper. The glass rod, which is sealed to the top of the semispheric ground joint and which ends closely above the water seal of the distilling flask, prevents boiling over.

(3) The receiving vessel (B), a 50 ml. Erlenmeyer flask, is connected to the apparatus by a double clamp (S). One clamp holds the vessel, the second one is fixed to the condenser and can be moved so that the end of the distilling apparatus, according to requirement, is immersed into the received liquid or ends freely above it. To facilitate work during the distillation a hinge is fixed on the clamp carrier, thereby putting the flask in an oblique position and making it possible to shake it backwards and forwards as well.

### *Reagents*

(1) Diluted phosphoric acid solution. 2-3 drops of concentrated  $H_3PO_4$  to 100 ml. of distilled water.

(2) Iodine solution. 0.75 ml. of 1/10 N iodine solution and 5 ml. of 20 per cent NaOH are filled up to 50 ml. with distilled water (prepared daily).

(3) potassium iodide crystals

(4) HCl concentrated diluted with equal parts of water in a small dropping bottle

(5) 1/1000 N sodium thiosulfate solution

(6) 0.25 per cent starch solution

### *Procedure*

Before beginning the determination, the distilling apparatus is rinsed with distilled water through the side tube (C) and the opening is closed quickly with the stopper, so that a small column of liquid in the capillary (D) closes the latter against the inside of the distilling headpiece. The ground neck part of the flask (A) is moistened with a bit of water. By means of the elastic springs the flask is fixed on the semisphere of the distilling headpiece. On the other side of the apparatus a flask (B) is clamped. Then water is heated in A and distilled into B until cleanliness of the apparatus is fully assured. Now the analysis is started with the determination of the 'blank value'.

A receiving flask B containing exactly 2 ml of the iodine solution (2) is fixed to the apparatus so that the distilling tube is immersed in the fluid. On the other side a distilling flask (A) containing 4 ml. of phosphoric acid (1) (measured with a small volumetric cylinder) is fixed in the manner explained above and is heated by a small flame. When the contents of the flask have boiled for three minutes flask (B) is pushed down so that the distilling tube is no longer immersed and boiling is continued for another minute only. Eventually distilled water is rinsed through the opening (C).

Some crystals of potassium iodide 1.5 ml. of 50 per cent HCl and a few drops of starch solution are put into flask (B). It is shaken and then titrated with 1/N 1000 sodium thiosulfate solution. The value thus found is the 'blank value'. In the same way potassium iodide HCl and starch solution are added to 2 ml. of iodine solution in a receiving flask and titration is carried out without distillation. The value obtained from this titration must be equal to the 'blank value'. When this is not the case the distillation must be repeated. Once the 'blank value' is found the actual determination can be carried out in succession. This is done by adding 0.2 ml. of blood or serum to be examined to the measured amount of phosphoric acid in the flask (A) the flask being attached to the apparatus in succession. Precautions necessary in exact analytical determinations have to be observed. Distillation is carried out in the same manner as described for the determination of the blank value. Titration of the iodine solution in flask (B), carried out subsequently, gives the full values.

### Calculation

'Blank value' minus sample value multiplied by 4.83 gives the acetone content in mg. per cent.

### Example

Blank value	2.72
Sample value	1.34
	<hr/>
Difference	1.38

$$1.38 \times 4.83 = 6.67 \text{ mg. per cent acetone.}$$

The figures found in table 10 are divided by 10 to give the acetone content in mg. per cent.

The normal values for acetone + acetoacetic acid vary between 1.5 and 2.5 mg per cent. Carbohydrate metabolism and fat degradation are closely interrelated. Ketobody formation is connected with a certain glucose requirement of the organism. In order to get complete oxydation of 1.5 Gm. of fatty acid the metabolism of 1 Gm. of glucose in the tissue is necessary. If the ratio of fatty acid to glucose is more than 1.5 an inhibition of complete fatty acid metabolism and an accumulation of ketobodies will occur. Increased ketobodies are found

- (1) during periods of starvation, caused by deficiency of carbohydrate
- (2) during normal pregnancy, and during toxic pregnancies
- (3) in ether and chloroform anaesthesia
- (4) in diabetes mellitus values up to 3.0 mg per cent in coma
- (5) in all processes which are accompanied by alkalosis (hyperventilation ileus, excessive alkali intake) an increase of ketobodies may occur, caused by elimination of acid from the tissues
- (6) intestinal obstruction

#### DETERMINATION OF ACETONBODIES AND $\beta$ -OXY BUTYRIC ACID<sup>1</sup>

*Principle of the method* In the deproteinized blood preformed acetone and acetone from acetoacetic acid are determined first then the acetone which has been obtained from  $\beta$  oxy butyric acid by chromic acid oxydation

#### Reagents

- (1) 5 per cent ammonia
- (2) a. 1 per cent alum solution    b. saturated lead acetate solution
- (3) dilute sulfuric acid 20 ml. of concentrated sulfuric acid are poured into water and after cooling made up to 100 ml.
- (4) chromic acid sulfuric acid 2 Gm. of potassium dichromate and 20 ml. of  $H_2SO_4$  concentration are dissolved in water and made up to 100 ml.
- (5) 20 per cent NaOH purest grade
- (6) N/100 iodine solution

*Procedure*

In a 250 ml volumetric flask 2.5 ml of oxalated blood is mixed with 1 ml of ammonia solution (1). The mixture is diluted with 150 ml of water then precipitated first with 5 ml of lead acetate and then with 10 ml of 1 per cent alum solution whereupon the volume is made up to the mark with water. After one-half hour the liquid is filtered through a double folded filter. 200 ml of protein free and carbohydrate free filtrate corresponding to 2 ml of blood are diluted with 100 ml of water. 2 ml of  $\text{H}_2\text{SO}_4$  and some talcum are added and the distillation carried out for ten minutes. The distilling flask (volume approximately 700 ml) is placed upon an asbestos-lined metal funnel (Babo funnel) and is connected with a descending condenser (length approximately 60 cm) by means of a bent glass tube and rubber stoppers (must be in very good condition). The distilling flask is equipped with a stopper into which a 100 ml dropping funnel is inserted. The receiving flask is well cooled with water and changed when 50 ml of chromic acid-sulfuric acid (4) are added slowly from the dropping funnel. The distillation is continued for twenty five minutes. In both distillates the acetone content is determined with N/100 iodine solution. To this purpose NaOH is added to 10 ml of N/100 iodine solution until the brown color disappears and the mixture is allowed to stand for ten minutes. It is then acidified with 25 per cent  $\text{H}_2\text{SO}_4$  (3) with cooling and immediately titrated with N/100 sodium thiosulfate solution. (The lower part of the condenser carries a 2-hole rubber stopper. A small glass tube bent at an angle with a Peligot tube attached is fitted through one hole. A rubber stopper of suitable size must be chosen to establish airtight connection with a 300 ml Erlenmeyer flask serving as receiver).

*Calculation*

In the first distillate a 94.4 per cent yield of the total acetone is obtained. Consequently 1 ml of N/100 iodine solution corresponds to  $0.0967 \times \frac{100}{94.4} = 0.1024$  mg of total acetone. Multiplication of the amount of iodine solution used by the factor 0.012 (as 2 ml of blood have been used for the analysis) gives the amount of total acetone in 1 ml of blood. If the amount of iodine used (or thiosulfate)



is multiplied by 5.12 the acetone content expressed in mg per cent is obtained. The second distillate averages set a yield of 69.2 per cent of acetone from  $\beta$ -oxy butyric acid. Consequently 1 ml of N/100 iodine solution corresponds to  $\frac{104.06 \text{ (mol weight of } \beta\text{-oxy butyric acid)}}{6 \times \frac{69.2}{100}}$

$\frac{104.06}{4.15} = 25 \text{ mg}$  1 ml of N/100 iodine solution corresponds to  $\frac{25}{100} = 0.25 \text{ mg}$  of butyric acid. Multiplication of the amount of

iodine solution used by 12.5 gives  $\beta$ -oxybutyric acid in mg per cent. In order to determine the total acetone bodies in the form of acetone, 200 ml of blood filtrate is diluted with 50 ml of water 2 ml of 25 per cent  $\text{H}_2\text{SO}_4$  and some talcum is added, and the distillation is started. As soon as boiling begins, 50 ml of chromic acid-sulfuric acid is added from a dropping funnel as described above. The distillation is continued for twenty five minutes. One ml of N/100 iodine solution corresponds to 0.22 mg of acetone bodies, calculated as acetoacetic acid. Multiplication of the amount of ml iodine solution used by the factor 11 gives the amount of acetone bodies in mg per cent. For each determination a blank run (test for purity of the reagents) must be carried out.

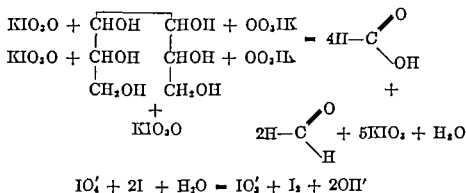
According to the authors this method gives satisfactory results.

In normal human blood 12 mg of  $\beta$ -oxy butyric acid per liter are found.

#### DETERMINATION OF POLY HYDROXY ALCOHOLS (SORBITOL MANNITOL) TOGETHER WITH GLUCOSE IN BLOOD\*

*Principle of the method* Two parallel determinations are performed in one analysis glucose is estimated according to Fujita Iwatake after deproteinizing with zinc hydroxide, in the other sample the sum of sugar and alcohol (sorbitose, mannose) is determined with periodate after deproteinizing with silicate. The difference of the values found equals the content of poly hydroxy alcohols.

# Reaction equation



# Reagent

For the determination of blood sugar according to Fujita Iwatake see p 247

For the periodate method (A) Deproteinizing

(1) stabilized silicic acid 15 Gm of sodium silicate ( $\text{Na}_2\text{SiO}_3 \times 8 \text{H}_2\text{O}$ ) are dissolved in 750 ml of distilled water and filtered through a fluted filter if cloudy To the clear solution 6 Gm of NaCl and 82 ml. of molar phosphoric acid are added and heated in a boiling water bath until a distinct opalescence is observed To the hot solution is added 60 ml of molar phosphoric acid (or see p 252)

(2) N NaOH

(B) for the oxydation

(3) (a) In a 1 liter volumetric flask 1 Gm of potassium periodate is dissolved in 800 ml of distilled water by placing the flask in a boiling water bath After cooling the volume is made up to the mark with distilled water

(b) 5 per cent sulfuric acid 20 ml of concentrated  $\text{H}_2\text{SO}_4$  (specific gravity 1.84) is poured into a 1 liter flask containing water, and is made up to the mark with water after cooling

Before use 3 parts of (a) and 2 parts of (b) are mixed (c) for the titration

(4) 12 per cent solution of secondary potassium phosphate in water

(5) KI crystalline purest grade

(6) N/200 sodium thiosulfate solution

(7) 0.25 per cent starch solution.

*Procedure*

The required amount of test tubes (plus 3 tubes for blank runs) is filled with 6 ml. of reagent (1) and 0.5 ml. of reagent (2). One tenth ml. (0.1 ml.) of whole blood (serum, plasma) is added to the sample tubes with the usual precautions. Then all tubes are immersed in a boiling water bath for three minutes. The coarse coagulated material floats on the surface of the liquid and must be well distributed by shaking before filtration. After cooling the contents are filtered through a funnel containing a small cotton plug serving as filter (The cotton must be washed with boiling water before use fig. 41). The tubes are rinsed three times with a small amount of hot water. Filtration is rapid and a clear filtrate is obtained.

To the deproteinized filtrate 5 ml. of acid periodate solution (3) are added, and the tubes are heated in a boiling water bath for twenty minutes. After cooling 4 ml. of secondary potassium phosphate solution (4) are added to each tube, well mixed, a small crystal of KI is added, the solution mixed again and the liberated iodine is titrated with N/200 thiosulfate solution till yellow and after the addition of starch solution the titration is continued till colorless.

*Calculation*

(Sugar + 6-valent alcohol) thiosulfate used up by the blank minus thiosulfate used up by the sample multiplied by 92 gives mg. per cent sugar plus alcohol.

The difference between this value and the sugar found in the determination according to Fujita Iwatake gives the content of 6-valent alcohol in the sample. The factor is calculated for the clinically occurring sugar and alcohol values.\*

*Example*

(a) blank value (Fujita Iwatake)	1.95 ml. N/200 thiosulfate solution
sample value (Fujita Iwatake)	<u>0.76 ml.</u> N/200 thiosulfate solution
difference	1.19 ml. corresponds to 207 mg. per cent sugar

\* For theoretical explanation see F. Rappaport, J. Reifer & H. Weinmann *Mikrochimica Acta* I 290 1937.

(b) blank value (periodate method)	4.72 ml N/200 thiosulfate solution
sample value (periodate method)	<u>1.15 ml</u> N/200 thiosulfate solution
difference	3.57 ml N/200 thiosulfate solution $\times 92 =$
sugar + alcohol value of 328 mg per cent	The alcohol content =
328 - 207 = 121 mg per cent	

This method has been found satisfactory for sorbitol and other hexanol tolerance tests in humans and animals

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## Chapter IX

# Fats and Lipids

All compounds which resemble fats in their physical and chemical properties, mainly in their characteristic solubility in organic solvents, are generally listed as lipids

The lipids can be subdivided into 3 groups

(1) free fatty acids and neutral fats (triglycerin esters tripalmitin tristearin, triolein)

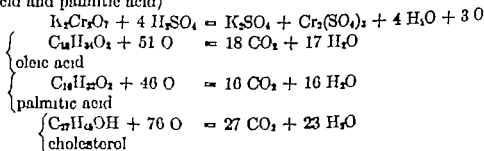
(2) Lipids which contain in their molecule nitrogen or nitrogen and phosphorus, such as phosphatides and cerebroside

(3) non saponifiable compounds some pigments, sterols and their esters

### DETERMINATION OF TOTAL LIPIDS BY PRECIPITATION AND EXTRACTION<sup>1</sup>

*Principle of the method* The blood fatty acids are extracted with alcohol-ether and oxydized in the heat by sulfuric acid potassium dichromate in the presence of ceric sulfate as catalyst. The excess potassium dichromate is determined iodometrically

*Reaction equation* (the fatty acids of blood consist mainly of oleic acid and palmitic acid)



### Reagents

(1) alcohol-ether mixture 3 parts of 95 per cent ethyl alcohol is mixed with 1 part of ether Both solvents must be redistilled before use to eliminate any impurities.

(2) sodium ethylate 2-3 Gm of metallic sodium is dissolved in 100 ml. of absolute alcohol with cooling The reagent will keep over

a period of time when stored in a cool and dark place. It must be discarded when it turns yellow.

(3) sulfuric acid: one part of concentrated  $H_2SO_4$  is poured into 3 parts of a 10 per cent sodium sulfate solution.

Reagents (2) and (3) must be compared with each other in the following way: one drop of phenolphthalein solution is added to 2 ml of freshly prepared sodium ethylate solution (2) and, using a serological pipet (fig. 4) the amount of  $H_2SO_4$  (3) is determined which neutralizes (decolorizes) the sodium ethylate solution. For the analysis a 10-15 per cent excess of this sulfuric acid is used.

(4) saturated chromic sulfate solution

(5) ceric sulfate-potassium dichromate reagent: 6 Gm. of ceric sulfate are suspended in 35 ml. of N/1 potassium dichromate solution + 151 ml. of water (the commercial ceric sulfate must be freed from acetic acid in a vacuum desiccator filled with  $CaCl_2$  and NaOH (solid), (see potassium determination p. 100). In order to dissolve the ceric sulfate 314 ml. of concentrated  $H_2SO_4$  are carefully added.

(6) petroleum ether (40-60°C). The petroleum ether must be redistilled, it is washed with concentrated  $H_2SO_4$  between distillations (fig. 40).

If the apparatus as shown in figure 40 is used, only one distillation is required. The outside, round bottom flask (A) is washed with concentrated sulfuric acid and filled with petroleum ether (chloroform or any suitable liquid may also be distilled in this manner). The distillation is carried out as usual. The petroleum ether distils over into the inside vessel (B). The constant heating caused by vapors entering from the tube (r) and by the steam inside the flask (A) effect a renewed distillation. The drainage tube (U) acts as a valve by causing the condensate which does not distil over during the second distillation, to run off and thus maintain a constant pressure inside the flask, assuring regular boiling. The inside vessel (B) is connected through the ground glass joint and a cork stopper with a distilling head (condenser and thermometer).

This apparatus is suitable for the redistillation of all organic solvents.

(7) chloroform (redistilled fig. 40)

(8) N/40 sodium thiosulfate solution

(9) 10 per cent KI solution freshly prepared

(10) 0.25 per cent starch solution

All glassware used in this method must be freed from fat or grease by soaking for twenty four hours in chromic acid sulfuric acid. After thorough rinsing with water, it should be dried in an oven at  $110^{\circ}\text{C}$ .

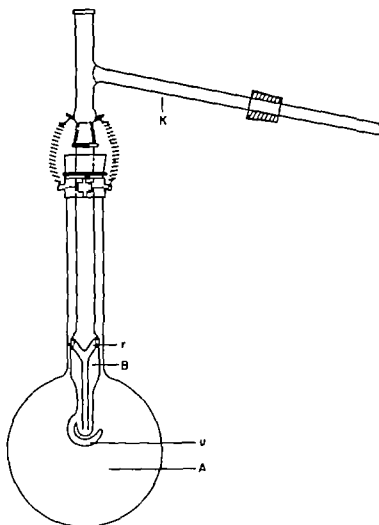


FIG. 49 APPARATUS FOR DOUBLE DISTILLATION IN ONE FLASK  
ACCORDING TO F. RAFFAPORT

### *Procedure*

With a capillary pipet 0.2 ml. of whole blood (serum) is drawn from the finger tip and delivered with shaking into a 20 ml. volumetric flask, containing approximately 15 ml. of the alcohol-ether mixture. The flask is immersed in a boiling water bath for thirty seconds during which time shaking is continued. After cooling down the volume

is made up to the mark with alcohol-ether. Now the mixture is filtered through a quantitative filter (Whatman 42 diameter 7 cm.) which has previously been boiled in alcohol. Fifteen ml. of filtrate is transferred to a long test tube with ground glass stopper (length 15-16 cm., inner diameter 2 cm.). If serum or oxalated blood is used for the analysis, one sample of 0.625 ml. of material in a 25 ml. volumetric flask may be deproteinized as described above instead of 3 samples of 0.2 ml. This sample is made up to the mark with alcohol-ether mixture filtered and 6 ml. of filtrate (corresponding to 0.15 ml. of blood) ( $\frac{25}{6} = \frac{0.625}{X}$   $X = 0.15$ ) is transferred to test tubes equipped with ground glass stoppers (length 13-14 cm., diameter approximately

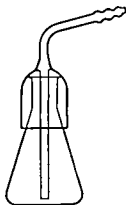


FIG. 50 BELL JAR FOR VACUUM EVAPORATION

22 mm.) After the addition of 0.5 ml. of sodium ethylate the mixture is concentrated on a water bath until a small amount of oily, gummy residue remains and no more odor of alcohol can be noticed. This residue is acidified with the previously determined amount of sulfuric acid (see preparation of reagent (3)) placed into a boiling water bath for 1 minute cooled and one drop of saturated chromic sulfate solution is added (to separate the petroleum ether and the aqueous layer). After the addition of 2.5 ml. of chloroform the tube is stoppered and shaken carefully but vigorously for 1 minute. Now 10 ml. of petroleum ether is added and the tube is again shaken for 1 minute. When the 2 layers have separated 10 ml. of the chloroform petroleum ether layer is transferred to a 150 ml. Erlenmeyer



flask. It is advisable to keep the tubes immersed in cold water during the entire procedure of shaking and separation. Petroleum ether and chloroform are evaporated from a hot plate and the last traces of solvent removed with a water pump using the apparatus shown in fig 50. After the addition of 10 ml of conc sulfuric acid-potassium dichromate reagent (if possible with an automatic pipet, see appendix, fig 60) the flask is covered with small glass dishes and heated in an oven at 120°C for twenty minutes to accomplish the oxidation. Now 75 ml of tap water is added to the hot flask. After cooling, 5 ml of potassium iodide solution are added and the liberated iodine titrated to yellow with N/40 sodium thiosulfate. Then a few drops of starch are added and the titration is continued until a pale green color appears.

### Calculation

The reaction equation shows that 1 ml of N/40 thiosulfate corresponds to  $\frac{2.6}{92 \times 40 \times 1000}$  g = 0.0008 mg of palmitic acid or  $\frac{2.82}{102 \times 40 \times 1000}$  g = 0.00091 mg of oleic acid. As compounds other than oleic and palmitic acid occur in blood, which also react with potassium dichromate and which belong to the lipid group, Blood assumes an average factor for 1 ml of N/40 thiosulfate = 0.000925 mg of lipid. For the analysis 0.2 ml of blood were made up to 20 ml, 15 ml of filtrate, corresponding to 0.15 ml of blood were made up to 12.5 ml during the course of the procedure. Ten ml of this dilution = 0.12 ml of starting material  $\left( \frac{10 \times 0.15}{12.5} \right)$  were oxidized with potassium dichromate. If the lipid content of 100 ml of starting material is to be calculated, the number of ml of dichromate (difference of thiosulfate used for the blank and for the sample) must be multiplied by the factor 0.000925, then divided by 0.12 and multiplied by 100. One ml of N/40 thiosulfate corresponds to  $\frac{0.000925 \times 100}{0.12} = 57.71$  mg per cent lipid (table 20).

TABLE 20—Table for the calculation of fat content

All N/40  $\text{Na}_2\text{S}_2\text{O}_3$  = mg % fat

	0.0	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80	0.90
		5 77	11 54	17 31	23 08	28 85	34 63	40 40	46 17	51 94
1	57 71	63 48	69 25	75 02	80 79	86 57	92 34	98 11	103 88	109 65
2	115 42	121 19	126 96	132 73	138 50	144 28	150 05	155 82	161 59	167 36
3	173 13	178 90	184 67	190 44	196 21	201 99	207 76	213 53	219 30	225 07
4	230 84	236 61	242 38	248 15	253 92	259 70	265 47	271 24	277 01	282 78
5	288 55	294 32	300 10	305 86	311 63	317 41	323 18	328 95	334 72	340 49
6	346 26	352 03	357 80	363 57	369 34	375 12	380 89	386 66	392 43	398 20
7	403 97	409 74	415 51	421 28	427 05	432 83	438 60	444 37	450 14	455 91
8	461 63	467 45	473 22	478 99	484 76	490 51	496 31	502 08	507 85	513 62
9	510 39	515 16	520 93	526 70	532 47	538 25	544 02	549 79	555 56	561 33
10	577 10	582 87	588 64	594 41	600 18	605 95	611 73	617 50	623 27	629 04
11	634 81	640 58	646 35	652 12	657 89	663 67	669 44	675 21	680 98	686 75
12	692 52	698 29	704 06	709 83	715 60	721 38	727 15	732 92	738 69	744 46
13	750 23	756 00	761 77	767 54	773 31	779 09	784 86	790 63	796 40	801 17
14	807 04	813 71	819 48	825 25	831 02	836 80	842 57	848 31	854 11	859 88
15	866 65	871 42	877 19	882 96	888 73	894 51	900 28	906 05	911 82	917 59
16	923 36	929 13	934 90	940 67	946 44	952 22	957 99	963 76	969 53	975 30
17	981 07	986 84	992 61	998 38	1004 15	1009 93	1015 70	1021 47	1027 23	1033 01
18	1038 78	1044 55	1050 32	1056 09	1061 86	1067 64	1073 41	1079 18	1084 95	1090 72
19	1096 49	1102 26	1108 03	1113 80	1119 57	1125 35	1131 12	1136 89	1142 66	1148 43
20	1154 20	1159 97	1165 74	1171 51	1177 28	1183 06	1188 83	1194 60	1200 37	1206 14
21	1211 91	1217 68	1223 45	1229 22	1234 99	1240 77	1246 54	1252 31	1258 08	1263 85
22	1260 62	1275 39	1281 16	1286 93	1292 70	1298 48	1304 25	1310 02	1315 79	1321 56
23	1327 33	1333 10	1338 87	1344 64	1350 41	1356 19	1361 96	1367 73	1373 50	1379 27
24	1385 04	1390 81	1396 58	1402 35	1408 12	1413 90	1419 67	1425 44	1431 21	1436 98
25	1442 75	1448 52	1454 29	1460 06	1465 83	1471 61	1477 38	1483 15	1488 92	1494 69
26	1500 46	1506 23	1512 00	1517 77	1523 51	1529 32	1535 09	1540 86	1546 63	1552 40
27	1558 17	1563 94	1569 71	1575 48	1581 25	1587 03	1592 80	1598 57	1604 31	1610 11
28	1616 83	1621 65	1627 42	1633 19	1638 96	1644 74	1650 51	1656 28	1662 05	1667 82
29	1673 00	1679 36	1685 13	1690 90	1696 67	1702 45	1708 22	1713 99	1719 75	1725 53

*Example*

thiosulfate used up by the blank	33.4 ml
thiosulfate used up by the sample	21.9 ml
difference	11.5 ml
11.5 ml $\times$ 57.71 = 663.66 mg per cent total lipids	

### DIRECT DETERMINATION OF TOTAL LIPIDS WITHOUT DEPROTEINIZING<sup>3</sup>

*Principle of the method* The sample (serum, whole blood tissue) is hydrolyzed with sodium ethylate in the Arta pot (fig 52) at 3 atmospheres pressure and extracted in acid medium with chloroform petroleum ether. The extract is concentrated and—as previously described—oxidized in the heat with sulfuric acid potassium dichromate in the presence of ceric sulfate as catalyst. The excess unchanged potassium dichromate is determined iodometrically.

#### *Reagents*

(1) sodium ethylate 1 Gm. of metallic sodium is dissolved in 50 ml. of absolute ethyl alcohol. This reagent will keep over a period of time when stored in a cool and dark place. It must be discarded when a yellow color is noted.

(2) sulfuric acid one part of concentrated  $H_2SO_4$  is diluted with 3 parts of a 10 per cent solution of sodium sulfate.

Reagents (1) and (2) are compared as follows. One drop of phenolphthalein solution is added to 3 ml. of freshly prepared sodium ethylate solution (2) and using a serologic pipet (fig 4) the number of ml. of  $H_2SO_4$  is determined which is needed to neutralize the sodium ethylate solution (decoloring). A 10–15 per cent excess of acid is used for the analysis.

(3) 10 per cent chromic sulfate solution

(4) ceric sulfate-potassium dichromate reagent 6 Gm. of ceric sulfate are suspended in 35 ml. of N/1 potassium dichromate solution and 151 ml. of water (commercially available ceric sulfate must be freed from acetic acid in a vacuum desiccator over  $CaCl_2$  and solid NaOH see p 109). This suspension is dissolved by careful addition of 314 ml. of concentrated  $H_2SO_4$ .

(5) petroleum ether (b.p. 40–60 C) The petroleum ether must be redistilled. Before the second distillation it is washed with concentrated sulfuric acid (fig 49).

(6) chloroform (redistilled fig 49)

(7) N/40 sodium thiosulfate solution

(8) 10 per cent potassium iodide solution freshly prepared

(9) 0.25 per cent starch solution

All glassware used must be freed from fat by soaking in chromic acid sulfuric acid for 24 hours, then rinsed with water and used when completely dry

### *Procedure*

The required number of test tubes (length 15-16 cm diameter approximately 2 cm ) with ground glass stoppers is filled with 0.5 ml of water. Two tenth ml (0.2 ml ) of whole blood serum or tissue (50-100 mg of tissue according to the amount of lipid expected) and 2 ml of sodium ethylate solution (1) are added. Two to three tubes serving as blanks and containing the same amount of reagents but no sample are treated in the same manner. All tubes are placed into a metal rack and heated in an Arta pot (fig 52) for 55 minutes at 130°C. The desired temperature is reached when the valve knob is adjusted to the - 3 mark. The time interval for heating up to 130° and the cooling-off period are not included in the 55 minutes. After cooling the previously determined amount of sulfuric acid (2) is added and when the examined material is colorless a drop of saturated chromic sulfate (for the separation of the aqueous layer from the petroleum ether layer) is added. Now 2.5 ml of chloroform are filled into each tube and the tubes are shaken several times. Ten milliliters of petroleum ether are added the tubes are tightly stoppered and shaken vigorously for several minutes. With some practice 6 tubes may easily be shaken simultaneously. The tubes are placed into cold water bath. After the layers have separated 10 ml of chloroform petroleum ether layer are removed and the solvent is evaporated from a hot plate or a small water bath (beware of open flame). The last traces of solvent are removed on a water pump using the apparatus shown in fig 50 the flask itself being immersed in hot water during that time. Great care must be taken to remove all traces of solvent as they often cause error in the results and also are responsible for badly checking controls. Now 10 ml of ceric sulfate-potassium dichromate solution (4) are added the flasks are covered with small beakers or glass dishes and placed into an oven at 120°C for twenty minutes to accomplish the oxydation. Seventy five (75) ml of tap water are added to the hot flasks. After cooling 5 ml. of KI solution (8) are added and the liberated iodine is titrated to yellow with thio-

sulfate and upon addition of a few drops of starch the titration is continued till the color changes to a pale green

TABLE 21—Table for the calculation of fat content

1 ml of N/40  $\text{Na}_2\text{S}_2\text{O}_3$  = mg % fat

	00	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0		4 32	8 64	12 06	17 28	21 60	25 92	30 24	34 56	39 88
1	43 20	47 52	51 84	56 16	60 48	64 80	69 12	73 44	77 76	82 08
2	86 40	90 72	95 04	99 36	103 7	103 0	112 3	116 6	121 0	125 3
3	129 0	133 0	138 2	142 6	146 9	151 2	155 5	159 8	164 2	168 4
4	172 8	177 1	181 4	185 8	190 1	194 4	193 7	203 0	207 4	211 7
5	216 0	220 3	224 6	228 7	233 0	237 3	241 6	246 9	250 3	254 6
6	259 2	263 5	267 8	272 8	276 5	280 8	285 1	289 4	293 8	298 1
7	293 4	306 7	311 0	315 4	319 7	324 0	323 3	332 6	336 9	341 2
8	345 6	349 0	354 2	358 6	362 9	367 2	371 5	375 8	380 2	384 5
9	388 8	393 1	397 4	401 8	406 1	410 4	414 7	419 0	423 4	427 7
10	432 0	436 2	440 6	445 0	449 3	453 6	457 9	462 2	466 6	470 9
11	475 2	479 5	483 8	488 2	492 5	496 8	501 1	505 4	509 8	514 1
12	518 4	522 7	527 0	531 4	535 7	540 0	544 3	548 6	553 0	557 3
13	561 6	565 0	570 2	574 6	579 0	583 3	587 6	591 0	596 3	600 6
14	604 8	609 1	613 4	617 8	622 1	626 4	630 7	635 0	639 4	643 7
15	648 0	652 3	656 6	661 0	665 3	669 6	673 0	678 2	682 6	686 9
16	691 2	695 5	699 8	704 2	708 5	712 8	717 1	721 4	725 8	730 1
17	734 4	738 7	743 0	747 4	751 7	755 0	760 3	764 6	768 0	773 3
18	777 6	781 9	786 2	790 6	794 0	799 2	803 5	807 8	812 6	816 5
19	820 8	825 1	829 4	833 8	838 1	842 4	846 7	851 0	855 4	859 7
20	864 0	868 3	872 6	877 0	881 3	885 6	890 0	894 2	898 6	902 9
21	907 2	911 5	915 8	920 2	924 5	928 8	933 1	937 4	941 8	946 1
22	950 4	954 7	959 0	963 4	967 7	972 0	976 3	980 6	985 0	989 3
23	993 6	998 0	1002 3	1006 6	1010 0	1015 2	1019 5	1023 8	1028 2	1032 5
24	1036 8	1041 1	1045 4	1049 8	1054 1	1058 4	1062 7	1067 0	1071 4	1075 7
25	1080 0	1084 3	1088 6	1093 0	1097 3	1101 6	1105 9	1110 3	1114 6	1118 9
26	1123 2	1127 5	1131 8	1136 2	1140 5	1144 8	1149 1	1153 4	1157 8	1162 1
27	1166 4	1170 7	1175 0	1179 3	1183 7	1188 0	1192 3	1196 6	1201 0	1205 3
28	1210 6	1215 0	1219 2	1223 6	1227 9	1232 3	1236 5	1240 8	1245 2	1249 5
29	1253 8	1258 1	1262 4	1266 8	1271 1	1275 4	1279 7	1284 0	1288 4	1292 7

### Calculation

The average factor of Bloor is used for the calculation of the fat content of tissue or blood, 1 ml of N/40 thiosulfate corresponding to 0.06925 mg of lipid. During the course of the determination 0.2 ml.

of blood was diluted to 12.5 ml. Ten milliliters of this dilution (= 0.16 ml of starting material  $\frac{10 \times 0.2}{12.5} = 0.16$ ) was oxidized with dichromate. If the lipid content in 100 ml. of original material is to be determined the following calculation must be carried out: number of ml. potassium dichromate (thiosulfate required by the blank minus thiosulfate required by the sample) multiplied by the factor 0.03925 divided by 0.16 and multiplied by 100. Consequently 1 ml. of N/40 sodium thiosulfate corresponds to  $\frac{0.06925 \times 100}{0.16} = 43.2$  mg. of lipid (table 21).

#### Example

thiosulfate used up by the blank	33.8 ml
thiosulfate used up by the sample	19.1 ml
difference	14.7 ml
$14.7 \text{ ml} \times 43.2 = 635.04 \text{ mg per cent total lipid}$	

Normally the total lipid content of serum (whole blood) varies between 500-750 mg per cent. Hyperlipemia occurs

- (1) during pregnancy and menstruation
- (2) in kidney diseases (nephrosis mainly in lipid nephrosis rarely in glomerulonephritis)
- (3) in anemias
- (4) in diabetes mellitus
- (5) in hypothyroidism
- (6) in fat transport (oral fat intake and mobilization of depot fat during fasting periods)
- (7) ether narcosis, alcoholism

A decreased lipid level has occasionally been encountered in hyperthyroidism tendency in schizophrenia.

#### DETERMINATION OF LECITHIN AND PHOSPHATIDES

Lecithin forms the largest part of the phosphatides. Therefore the lecithin formula may be used for the calculation of both lecithin and phosphatides. The lecithin content of blood (serum) is calculated from the lipid phosphorus (chapter III p. 99) by multiplication with 25. Lecithin contains approximately 4 per cent phosphorus.

*Example*

If 9.09 mg of lipid phosphorus have been found the lecithin content of blood is  $9.09 \times 25 = 227.25$  mg per cent

The normal lecithin values vary between 175 and 300 mg per cent.

### COLORIMETRIC DETERMINATION OF CHOLESTEROL<sup>4</sup>

(1) colorimetric determination of total cholesterol

*Principle of the method* In the presence of concentrated  $\text{H}_2\text{SO}_4$  cholesterol gives a green color with acetic anhydride. The intensity of this color is directly proportional to the cholesterol content of the sample. The colors are compared in a Hellige or Dubosque colorimeter

#### *Determination of Total Cholesterol*

*Reagents*

(1)  $\text{NaOH}$  c.p. pellets (Merck)

(2) chloroform

(3) kaolin ( $\text{H}_2\text{Al}_2\text{Si}_2\text{O}_5 \times \text{H}_2\text{O}$ ) (Bolus alba)

(4) acetic anhydride

(5) concentrated  $\text{H}_2\text{SO}_4$

(6) cholesterol standard stock solution 100 mg of pure cholesterol is dissolved in 100 ml of redistilled chloroform

( $\alpha$ ) working standard for the Dubosque colorimeter in a 100 ml graduated cylinder 4 ml of stock solution are made up to the mark with chloroform

( $\beta$ ) working standard for the Hellige colorimeter in a 100 ml graduated cylinder 10 ml of stock solution are made up to the mark with chloroform

As chloroform is very volatile it is recommended to keep both stock solution and working standard in high cylinders with ground glass stoppers and to note the chloroform level. Before use any evaporated chloroform should be replaced. Both solutions must be kept in the refrigerator in a dark place as cholesterol dissolved in chloroform can be destroyed by sunlight

*Procedure*

To test tubes equipped with ground glass top (length 13-16 cm) and containing 1.5 ml of water is added 0.2 ml of serum or whole blood with a capillary pipet and 2 pellets of  $\text{NaOH}$  corresponding to 0.4 Gm of  $\text{NaOH}$ . The tubes are shaken and warmed slightly to

dissolve the NaOH and are then placed into a boiling water bath for two hours.

This process can be accelerated by allowing the reaction to take place at a higher temperature.<sup>5</sup> To obtain this higher temperature the water bath is replaced by a glycerin bath (according to the physical law, a raise in temperature of 10 degrees C. speeds up the reaction by about  $2\frac{1}{2}$  times). If a glycerin bath of a temperature of  $123^{\circ}\text{C}$  is used the cholesterol is completely saponified in about five minutes.

After cooling 10 ml. of chloroform is added, the tubes are shaken vigorously for 1-2 minutes with outside cooling. Approximately 3 Gm. of kaolin is now added (to absorb the water) the tubes are again shaken until the precipitate forms small balls and the mixture is filtered. To 5 ml. of filtrate 1 ml. of acetic anhydride<sup>6</sup> and 0.1 ml. of  $\text{H}_2\text{SO}_4$  are added and the tubes are placed into an incubator at  $37^{\circ}\text{C}$  for fifteen minutes. If no incubator is available the tubes may be left for twenty minutes at room temperature in a dark place. Now the colors are compared in a colorimeter.

When the Dubosque colorimeter is used 5 ml. of dilute working standard solution (6a) are treated as above described for the sample.

When the Hellgo colorimeter is used 10 ml. of dilute standard (6b) are treated together with the blood extract. 2 ml. of acetic anhydride and 0.2 ml. of  $\text{H}_2\text{SO}_4$  being added. Since the green color is not stable a separate standard tube must be set up for each unknown sample keeping the time interval constant.

The colors obtained with this method may sometimes show a slight yellow tint which tends to make the comparison with the standard rather difficult. Uniform colors may be obtained when following the directions of Sperry.<sup>7</sup> With ice cooling 0.1 volume of concentrated  $\text{H}_2\text{SO}_4$  is added drop by drop to the required volume of acetic anhydride (i.e. 3 ml. of acetic anhydride + 0.3 ml. of  $\text{H}_2\text{SO}_4$ ). Now 1.1 ml. of acid-anhydride mixture are added to 5 ml. of chloroform extract or standard solution avoiding any contamination with water. The colors are compared after the time interval indicated above. The laborious pipetting of small amounts of  $\text{H}_2\text{SO}_4$  is thus omitted.

#### Calculation

(a) for the Dubosque colorimeter

$$\frac{\text{reading of standard} \times 0.2 \times 1000}{\text{reading of unknown}} = \text{mg. per cent cholesterol}$$



*Calculation*

See total cholesterol, p. 297

INDIRECT TITRIMETRIC DETERMINATION OF CHOLESTEROL  
DIGITONIN<sup>1, 2</sup>*Determination of Total Cholesterol*

*Principle of the method* With the addition of a known amount of digitonin solution the cholesterol is precipitated as digitonide. Excess digitonin is hydrolyzed and the liberated hexoses are determined according to Hagedorn-Jensen. If whole blood or serum is used for the analysis of total cholesterol, the esters must be saponified with alcoholic NaOH under 3 atm. pressure.

*Reagents*

(1) sodium ethylate solution. 1 Gm. of metallic sodium is dissolved in 50 ml. of absolute alcohol with cooling. The reagent should be freshly prepared before use.

(2) chloroform. commercially available chloroform must be distilled (fig. 40). After the distillation the chloroform is washed 3 times with 3 times its volume of water in a separatory funnel, the aqueous (top) layer being removed each time with the apparatus shown in fig. 51<sup>10</sup>. The chloroform is then dried with kaolin, filtered through a fluted filter. If redistilled chloroform has been kept over a prolonged period of time it must be washed again.

(3) 0.85 per cent NaCl solution

(4) kaolin (bolus alba) Merck

(5) digitonin solution. In a 50 ml. volumetric flask 33 mg. of digitonin are added to some absolute ethyl alcohol and dissolved by heating in a boiling water bath. After cooling 16.7 ml. of distilled water is added. It is made up to the mark with alcohol and filtered. This digitonin solution will keep indefinitely when stored in a cool and dark place. (When pure cholesterol solutions are analyzed, the digitonin solution must be freshly prepared each time.)

(6) 40 per cent sulfuric acid. prepared by pouring 108 ml. of concentrated  $H_2SO_4$  into 400 ml. of distilled water.

(7) 8-9 per cent NaOH. prepared from sodium hydroxide c.p.

(8) 0.1 per cent alcoholic phenolphthalein solution

(9) buffered potassium ferricyanide solution according to Fujita Akiji & Danzo Iwatake (see p 247)

(a) 5.04 Gm of potassium ferricyanide is dissolved in 1 liter of distilled water

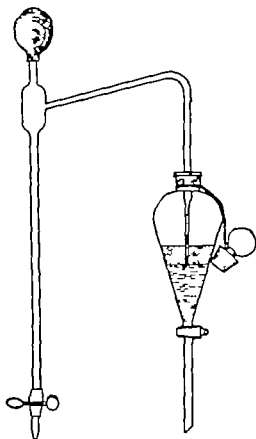


FIG 51 Separatory funnel with syphon according to Jacobsen and Dinmore

(b) 101 Gm of secondary potassium phosphate and 33.6 Gm tertiary potassium phosphate are dissolved in water and made up to 1 liter. Before use equal parts of (a) and (b) are mixed

(10) zinc sulfate-potassium iodide solution (see glucose determination according to Hagedorn-Jensen p 243)

(11) HCl solution 20 ml of fuming HCl are diluted with 80 ml of water

(12) N/200 sodium thiosulfate solution

(13) 0.25 per cent starch solution

### Procedure

With a calibrated capillary pipet 0.2 ml. of whole blood is drawn from the finger tip (or 0.2 ml. of oxalated blood, serum or plasma is used) and after wiping the outside of the pipet the sample is slowly delivered into a test tube with ground glass stopper (length 13–13.5 cm. diameter 2.2 cm.), containing 2 ml. of sodium ethylate solution. Determinations should be run in duplicate or triplicate and each series must also contain 2–3 blank runs. For the blank run, the

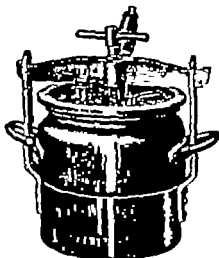


FIG. 52 ARTA POT

tubes are filled with 0.2 ml. of 0.85 per cent NaCl solution and 2 ml. of sodium ethylate solution. Blank tubes and sample tubes are placed into a metal frame and heated in a steam pot for 55 minutes at  $130^{\circ}\text{C}$ . We have used Arta pots (fig. 52) which give the desired pressure by adjustment to the third valve knob. The time required for heating up to the desired temperature and the time for cooling down is not calculated in the 55 minutes. After cooling the contents of each tube are extracted with 10 ml. of chloroform each, shaking vigorously. Approximately 1 Gm. of kaolin is added to each tube to absorb the water, the tube is again shaken and the sediment is allowed to settle. If the supernatant liquid is still cloudy, it is again

shaken, and filtered (Whatman 42 diameter 7 cm) Five ml of filtrate are transferred to test tubes bearing a mark at 12 ml (diameter 10-18 mm length 100 mm) The chloroform is evaporated from a hot water bath (an open flame is to be avoided as phosgene formation will interfere with the determination) The last traces of chloroform are removed with a water pump (fig 50) the tubes being placed into hot water during the procedure To each tube is now added 1 ml of absolute alcohol and all tubes are immersed in a hot water bath for a short period of time to dissolve the residue Now each tube receives 3 ml of digitonin solution (6) and the tubes are again placed into the boiling water bath for five minutes the flame being removed The tubes are cooled, 2 ml of water of 60°C. are added and the tubes are shaken vigorously until flocculation occurs Now the tubes are left to stand over night in a cool and dark place they are then filled up to the mark with water well mixed with a capillary pipet and filtered (Whatman No 42, diameter 7 cm) care being taken that the rough side of the filter paper is turned inside.

One ml of  $H_2SO_4$  (6) is added to 10 ml of filtrate in a Hagedorn Jensen tube The filtrate is hydrolyzed for two hours in a current of steam at 102°C or for 3 hours in the Arta pot pressure adjustment in the middle between mark 1 and 2 It is recommended to cover the tubes with small funnels or glass dishes After cooling the walls of the funnels or dishes are rinsed with a small amount of distilled water 0.1 ml of phenolphthalein is added and the hydrolysate neutralized with NaOH (7) Now 5 ml of potassium ferrieyanide mixture are added and the tubes are heated in a boiling water bath as usual After cooling 2 ml of HCl (11) and 2 ml of zinc sulfate-potassium iodide solution (10) is added and the liberated iodine is titrated with  $N/200$  sodium thiosulfate starch being used as indicator

### Calculation

Thiosulfate required by the sample minus thiosulfate required by the blank multiplied by 115 (empirically determined factor) gives mg per cent cholesterol (table 22)

With this method and using 0.2 ml of whole blood serum or plasma cholesterol values up to 500 mg per cent can be determined If higher values are expected 0.1 ml of sample is analyzed

TABLE 22—Table for the calculation of cholesterol content  
 $M \Delta / 200 \Delta n_D^{20} = \text{mg } \% \text{ cholesterol}$

	0	1	2	3	4	5	6	7	8	9
0 0		1 15	2 30	3 45	4 60	5 75	6 90	8 05	9 20	10 35
0 1	11 50	12 65	13 80	14 95	16 10	17 25	18 40	19 55	20 70	21 85
0 2	23 00	24 15	25 30	26 45	27 60	28 75	29 90	31 05	32 20	33 35
0 3	34 50	35 65	36 80	37 95	39 10	40 25	41 40	42 55	43 70	44 85
0 4	46 00	47 15	48 30	49 45	50 60	51 75	52 90	54 05	55 20	56 35
0 5	57 50	58 65	59 80	60 95	62 10	63 25	64 40	65 55	66 70	67 85
0 6	69 00	70 15	71 30	72 45	73 60	74 75	75 90	77 05	78 20	79 35
0 7	80 50	81 65	82 80	83 95	85 10	86 25	87 40	88 55	89 70	90 85
0 8	92 00	93 15	94 30	95 45	96 60	97 75	98 90	100 05	101 20	102 35
0 9	103 50	104 65	105 80	106 95	108 10	109 25	110 40	111 55	112 70	113 85
1 0	116 00	116 15	117 30	118 45	119 60	120 75	121 90	123 05	124 20	125 35
1 1	126 50	127 65	128 80	129 95	131 10	132 25	133 40	134 55	135 70	136 85
1 2	138 00	139 15	140 30	141 45	142 60	143 75	144 90	146 05	147 20	148 35
1 3	149 50	150 65	151 80	152 95	154 10	155 25	156 40	157 55	158 70	159 85
1 4	161 00	162 15	163 30	164 45	165 60	166 75	167 90	169 05	170 20	171 35
1 5	172 50	173 65	174 80	175 95	177 10	178 25	179 40	180 55	181 70	182 85
1 6	184 00	185 15	186 30	187 45	188 60	189 75	190 90	192 05	193 20	194 35
1 7	196 50	197 65	198 80	199 95	201 10	202 25	203 40	204 55	205 70	206 85
1 8	207 00	208 15	209 30	210 45	211 60	212 75	213 90	215 05	216 20	217 35
1 9	218 50	219 65	220 80	221 95	223 10	224 25	225 40	226 55	227 70	228 85
2 0	230 00	231 15	232 30	233 45	234 60	235 75	236 90	238 05	239 20	240 35
2 1	241 50	242 65	243 80	244 95	246 10	247 25	248 40	249 55	250 70	251 85
2 2	253 00	254 15	255 30	256 45	257 60	258 75	259 90	261 05	262 20	263 35
2 3	264 50	265 65	266 80	267 95	269 10	270 25	271 40	272 55	273 70	274 85
2 4	276 00	277 15	278 30	279 45	280 60	281 75	282 90	284 05	285 20	286 35
2 5	287 50	288 65	289 80	290 95	292 10	293 25	294 40	295 55	296 70	297 85
2 6	299 00	300 15	301 30	302 45	303 60	304 75	305 90	307 05	308 20	309 35
2 7	310 50	311 65	312 80	313 95	315 10	316 25	317 40	318 55	319 70	320 85
2 8	322 00	323 15	324 30	325 45	326 60	327 75	328 90	330 05	331 20	332 35
2 9	333 50	334 65	335 80	336 95	338 10	339 25	340 40	341 55	342 70	343 85
3 0	346 00	346 15	347 30	348 45	349 60	350 75	351 90	353 05	354 20	355 35
3 1	356 50	357 65	358 80	359 95	361 10	362 25	363 40	364 55	365 70	366 85
3 2	368 00	369 15	370 30	371 45	372 60	373 75	374 90	376 05	377 20	378 35
3 3	379 50	380 65	381 80	382 95	384 10	385 25	386 40	387 55	388 70	389 85
3 4	391 00	392 15	393 30	394 45	395 60	396 75	397 90	399 05	400 20	401 35
3 5	402 50	403 65	404 80	405 95	407 10	408 25	409 40	410 55	411 70	412 85
3 6	414 00	415 15	416 30	417 45	418 60	419 75	420 90	422 05	423 20	424 35
3 7	425 50	426 65	427 80	428 95	430 10	431 25	432 40	433 55	434 70	435 85
3 8	437 00	438 15	439 30	440 45	441 60	442 75	443 90	445 05	446 20	447 35
3 9	449 50	449 65	450 80	451 95	453 10	454 25	455 40	456 55	457 70	458 85

Ml	4	5	6	7	8	9	10
mg%	400	575	690	805	920	1035	1150

*Example*

thio sulfate used up by the sample	3.39 ml
thio sulfate used up by the blank	1.08 ml
difference	<u>2.31 ml</u>
$2.31 \text{ ml} \times 115 = 265.6 \text{ mg per cent cholesterol}$	

*Determination of Free Cholesterol<sup>11</sup>*

*Principle of the method* The total lipids of blood are extracted with alcohol-ether the extract is evaporated to dryness on a water bath, the residue is made alkaline and the free cholesterol is extracted with chloroform. Then the free cholesterol is precipitated with digitonin (see determination of total cholesterol) the excess digitonin is hydrolyzed to hexoses and the hexoses are determined according to the method of Fujita Akiji and Danzo Iwatake.

*Reagents*

(1) alcohol-ether mixture 3 parts of 95 per cent alcohol are mixed with 1 part of ether. Both solvents must be redistilled before use (fig. 49)

(2) 2 per cent NaOH

(3) 0.5 per cent sodium sulfate solution

All other reagents see determination of total cholesterol p. 300

*Procedure*

The required number of test tubes plus 3 tubes for blank runs are filled with 5 ml of alcohol-ether mixture (1) each. With an exactly calibrated capillary pipet 0.2 ml of serum (plasma or whole blood) is measured out. The outside of the pipet is carefully wiped and the blood is added gradually and with shaking of the tube to the alcohol-ether mixture. The pipet is blown out to remove the last traces of sample. The tubes are immersed in boiling water until the mixture begins to boil and boiling continued for 30 seconds with constant shaking. After cooling to room temperature it is filtered into wide test tubes equipped with ground glass stoppers (see total cholesterol). As filter serves a plug of cotton, from which all fat has been removed by refluxing for several hours with 95 per cent alcohol (soxhlet). The

tubes are rinsed 3 times with a few ml of alcohol-ether mixture and the washings are also filtered. The solvent is removed from a water bath, which is slowly heated to boil. It is evaporated to dryness and the last traces of solvent are removed with a water pump (fig 30). Now the tubes are placed into a warm water bath (approximately  $60^{\circ}\text{C}$ ) for several minutes. To the warm tubes 0.5 ml of 2 per cent  $\text{NaOH}$  is added and they are again warmed up in the water bath for a few seconds. After cooling the residue is extracted with 10 ml of chloroform shaking for one minute, each tube receives 1 Gm. of kaolin to absorb the water. It is again shaken vigorously and filtered (Whatman No 42, diameter 7 cm). Five milliliters of filtrate are transferred to test tubes, bearing a mark at 12 ml. The chloroform is removed from a hot water bath (an open flame has to be avoided to prevent phosgene formation which will interfere with the determination). The last traces of chloroform are removed with suction on a water pump the tubes being immersed in hot water to speed up the removal of solvent. The residue is dissolved in 1 ml of absolute alcohol and all tubes are placed into the hot water bath for a short time for complete solution. Each tube receives 3 ml of digitonin solution and the tubes are immediately placed into a boiling water bath from which the flame has been removed. After cooling down to body temperature the cholesterol-digitonide is precipitated with 2 ml. of water of  $50^{\circ}\text{C}$ . The tubes are best left over night in a cool and dark place. Then they are filled up to the mark with 0.5 per cent sodium sulfate solution they are well mixed and filtered, care being taken that the rough side of the filter paper is turned to the inside (Whatman No 42). One ml of 40 per cent  $\text{H}_2\text{SO}_4$  is added to 10 ml of filtrate, the procedure is continued as described for the determination of total cholesterol.

### Calculation

Thiosulfate used up by the sample minus thiosulfate used up by the blank multiplied by 110 (empirically determined factor) gives free cholesterol in mg per cent. Total cholesterol - free cholesterol = cholesterol-esters.

*Example*

thiosulfate used up by the sample	2.27 ml
thiosulfate used up by the blank	1.00 ml
difference	$1.18 \text{ ml} \times 115 =$
free cholesterol content	135.7 mg per cent
total cholesterol (see p. 306)	265.6 mg per cent
cholesterol esters	129.9 mg per cent

### TITRIMETRIC DIRECT OXYDATIVE DETERMINATION OF TOTAL CHOLESTEROL<sup>13</sup>

*Principle of the method* The sample is hydrolyzed with sodium ethylate in the Arta pot at 3 atm pressure and after cooling is extracted in *alkaline* medium with chloroform-petroleum ether. The extract is concentrated and the residue oxydized in the heat with sulfuric acid potassium dichromate in the presence of ceric sulfate as catalyst. The excess of unchanged potassium dichromate is determined iodometrically.

*Reagents*

(1) sodium ethylate solution 1 Gm of metallic sodium is dissolved in 50 ml of absolute alcohol. This reagent will keep over a period of time when stored in a cool and dark place. When it turns yellow it must be discarded.

(2) 10 per cent chromic sulfate solution

(3) ceric sulfate-potassium dichromate reagent 1.01 ml of water are added to 30 ml of N/1 potassium dichromate solution and 6 Gm of ceric sulfate are suspended in the solution. The suspension is dissolved by carefully adding 314 ml of concentrated  $\text{H}_2\text{SO}_4$ .

(4) petroleum ether (b.p. 40–60°C) the petroleum ether must be redistilled. It is washed with concentrated  $\text{H}_2\text{SO}_4$  before the second distillation.

(5) chloroform redistilled

(6) N/40 sodium thiosulfate solution

(7) 10 per cent potassium iodide solution, freshly prepared

(8) 0.25 per cent starch solution

All glassware used must be freed from fat and grease by soaking



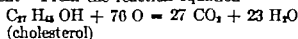
in chromic acid sulfuric acid for 24 hours, followed by rinsing and thorough drying

### *Procedure*

The required number of test tubes (length 15-16 cm diam 2 cm.) with ground glass stoppers are filled with 0.2 ml of water. To this is added 0.2 ml of whole blood or serum, measured with an exactly calibrated pipet and 2 ml of sodium ethylate (1) is added. An alternate method is to add the blood or serum directly to the sodium ethylate solution, which has been placed into the test tubes. (If this method is used, 0.2 ml of 0.85 per cent sodium chloride solution is added to the tubes representing the blank runs.) For each series of analyses 2 or 3 blank runs must be added which are heated in the same manner as described for the samples. All tubes are placed into a metal rack and heated in the Arta pot for fifty five minutes. The desired temperature is reached by adjusting the valve knob to the mark 3. The time required for heating up to the desired temperature and the time required for cooling down is not calculated in the fifty five minutes. After the tubes have been removed from the pot, each tube receives 0.2 ml of chromic sulfate solution (2) and the contents are well mixed (for serum only). After the addition of 2.5 ml of chloroform (5) the tubes are vigorously shaken for four minutes. Now 10 ml of petroleum ether (4) are added and after stoppering the tubes tightly they are again shaken for four minutes. Six tubes may be shaken simultaneously. Now the tubes are placed into the rack and immersed in cold water for the separation of the two layers. Ten ml of chloroform petroleum ether layer are transferred to an Erlenmeyer flask and the solvents are evaporated from a hot plate until a small amount of residue remains. The last traces of solvent are removed by a water pump, using a bell as shown in figure 50, the flask being immersed in hot water during the process. Great care must be taken to remove *all* solvent. To the residue 5 ml of ceric sulfate-potassium dichromate reagent (3) are added and the flasks are covered with a small beaker. The oxydation is performed in an oven at 120°C over a period of twenty minutes. Approximately 50 ml of tap water are added to the hot flasks. After cooling, 2 ml of KI solution (7) are added and the liberated iodine is titrated with thiosulfate (6) till yellow and after the addition of a few drops of starch the titration is continued till a pale green color appears.

*Calculation*

Thiosulfate required by the blank minus thiosulfate required by the sample multiplied by 39.7 gives cholesterol content of the sample in mg per cent. From the reaction equation



It may be seen that 1 ml. of N/40 sodium thiosulfate corresponds to  $\frac{386.37}{152 \times 40 \times 1000} = 0.0035$  mg. of cholesterol. For the analysis

0.2 ml. of blood was diluted to a volume of 12.5 ml. 10 ml. of this dilution, corresponding to 0.16 ml. of starting material =  $\frac{0.2 \times 10}{12.5}$  was

oxidized with dichromate. If the cholesterol content of the sample in 100 ml. of material is to be calculated, the number of ml. dichromate used (difference of thiosulfate between blank and sample) is multiplied by the factor 0.0035 divided by 0.16 and multiplied by 100. Consequently 1 ml. of N/40 thiosulfate corresponds to  $\frac{0.0035 \times 100}{0.16} = 39.7$  mg. per cent cholesterol (table 23)

*Example*

thiosulfate used up by the blank	16.7 ml
thiosulfate used up by the sample	9.8 ml
difference	6.9 ml
$6.9 \text{ ml} \times 39.7 = 273.9 \text{ mg. per cent total cholesterol}$	

The values found by this method agree well with the values found by the digitonin method.

Using 0.2 ml. of whole blood or serum this method permits the estimation of a cholesterol content up to 600 mg. per cent. If higher values are expected 0.1 ml. of sample are analyzed.

The total cholesterol content of serum or whole blood varies between 140-200 mg. per cent.

The content of cholesterol-esters in serum or plasma in normal persons is 60-70 per cent of the total cholesterol; the rest is free cholesterol. The determination of free cholesterol is best done in serum as the red cells contain almost only free cholesterol; therefore the fluctuations of free cholesterol in serum are not noted as distinctly when whole blood is used for the estimation.

Hypercholesterolemia is found in

(1) diabetes mellitus during naturally occurring as well as during experimental diabetes the cholesterol level rises (up to 1000 mg. per cent) Under the influence of insulin the cholesterol level rapidly returns to normal together with a return to normal of the glucose A drop of glucose without a simultaneous drop in cholesterol is to be regarded as clinically unfavorable

(2) lipid nephrosis values of 500-700 mg per cent are not rare. The increase is mainly in the cholesterol-ester fraction

TABLE 23

Ml N/40  $\text{Na}_2\text{S}_2\text{O}_3 \approx$  mg per cent cholesterol

ccm	0	1	2	3	4	5	6	7	8	9
0 0		3 07	7 04	11 01	15 88	19 85	23 82	27 79	31 76	35 73
1 0	39 70	43 67	47 04	51 61	55 58	59 55	63 52	67 49	71 46	75 43
2 0	79 10	83 37	87 34	91 31	95 28	99 25	103 2	107 2	111 2	115 2
3 0	119 1	123 1	127 0	131 0	135 0	138 0	143 0	147 0	151 0	154 8
4 0	158 8	162 8	166 7	170 7	174 7	178 7	182 6	186 6	190 6	194 5
5 0	198 3	202 5	206 4	210 4	214 4	218 3	222 3	226 3	230 3	234 2
6 0	238 2	242 2	246 1	250 1	254 1	258 1	262 0	266 0	270 0	274 0
7 0	277 9	281 9	285 4	289 8	293 8	297 7	301 7	305 7	309 7	313 6
8 0	317 0	321 0	325 5	329 5	333 5	337 5	341 4	345 4	349 4	353 3
9 0	357 3	361 3	365 2	369 2	373 2	377 2	381 1	385 1	389 1	393 0
10 0	397 0	401 0	404 9	408 9	412 9	416 8	420 8	424 8	428 8	432 7
11 0	436 7	440 7	444 0	448 0	452 6	456 0	460 5	464 5	468 5	472 4
12 0	476 4	480 1	484 3	488 3	492 3	496 2	500 2	504 2	508 2	512 1
13 0	516 1	520 1	524 0	528 0	532 0	536 0	539 9	543 9	547 9	551 8
14 0	555 8	559 8	563 7	567 7	571 7	575 6	579 6	583 0	587 6	591 5
15 0	595 5	599 5	604 1	607 4	611 4	615 4	619 3	623 3	627 3	631 2

(3) glomerulo nephritis occasionally an increased cholesterol level may be found in this disease

(4) pregnancy during the entire period of pregnancy the cholesterol level is high it returns to normal approximately three weeks post partum

(5) before menstruation

(6) arteriosclerosis

(7) Schueller-Christian's disease increase mainly in the ester fraction

(8) cirrhosis of the liver hypercholesterolemia with maximal increase of free cholesterol

(9) morbus Gaucheri

(10) Pick Nieman s disease

(11) myxedema (especially of children)

(12) senile cataract

(13) celiac disease of children

(14) psoriasis

Hypocholesterolemia is found in

(1) pernicious anemia

(2) hemolytic jaundice

(3) acute liver disease

(4) starvation

(5) acute infectious diseases

(6) hyperthyroidism

(7) congestive heart failure

(8) coronary artery thrombosis

(9) schizophrenia

(10) acute pancreatitis

## DETERMINATION OF UNSATURATED FATTY ACIDS AND OF THE IODINE NUMBER<sup>12</sup>

### *Determination of Unsaturated Fatty Acids*

*Principle of the method* The analytical sample (serum whole blood tissue) is hydrolyzed with sodium ethylate in the Arta pot at 3 atm pressure. The interfering cholesterol is removed in alkaline medium and after acidification the total fatty acids (saturated and unsaturated) are extracted with chloroform and petroleum ether. The extract is concentrated the residue taken up in glacial acetic acid and the fatty acids are saturated according to Rosemund and Kuhn<sup>13</sup> by the addition of bromine-pyridine sulfate. The excess bromine-pyridine sulfate is determined iodometrically.

### *Reagents*

(1) sodium ethylate solution 1 Gm. of metallic sodium is dissolved in 40 ml. of absolute alcohol with cooling. This reagent will keep over a period of time it must be discarded when it turns yellow.

(2) chloroform redistilled (fig. 49)

(3) petroleum ether (b p 40-60°C) the petroleum ether must be redistilled. It is washed with concentrated sulfuric acid before the second distillation (see fig 51)

(4) sulfuric acid, 1 part of concentrated  $H_2SO_4$  is poured into 3 parts of a 10 per cent solution of sodium sulfate

Reagents (1) and (4) must be compared as follows. One drop of phenolphthalein solution is added to 2 ml of freshly prepared sodium ethylate solution (1) and using a serological pipet (fig 4) the amount of sulfuric acid (4) is determined which is necessary to neutralize (decolorize) the ethylate solution. For the subsequent analysis a 10-15 per cent excess of acid is used

(5) bromine-pyridine reagent (a) 16 ml of pyridine (purest grade, Merck) is dissolved in 10 ml of glacial acetic acid

b 11 ml of concentrated  $H_2SO_4$  is added to 10 ml of glacial acetic acid

c 0.5 ml of bromine is dissolved in 10 ml of glacial acetic acid. All solutions are prepared with ice cooling. Solutions a) and b) are mixed, and solution c) is added last. The mixture is made up to approximately 200 ml with glacial acetic acid

(6) lithium bromide reagent 3 per cent solution of lithium bromide in glacial acetic acid. Approximately one part of (5) and 2 parts of (6) are mixed before use in such a way that 4 ml of the mixture use up approximately 6-8 ml of N/100 thiosulfate

(7) glacial acetic acid

(8) KI, crystalline

(9) 0.25 per cent starch solution

(10) N/100 sodium thiosulfate solution

### *Procedure*

The required number of test tubes (length 16 cm diameter 2 cm) equipped with ground glass stoppers are filled with 0.5 ml of water followed by 0.625 ml of serum (see later for whole blood and tissue) and 2 ml of sodium ethylate solution (1). The tubes are placed into the Arta pot (fig 52) for hydrolysis and are heated for 55 minutes at 130°C. the necessary pressure is obtained by adjusting the third valve knob. The time intervals required for heating up to 130° and for subsequent cooling down are not included in the above mentioned 55 minutes. Care must be taken to heat the pot slowly which

is filled with cold water. The pot should not be opened until it has completely cooled down, otherwise material may be lost by spattering. It is recommended to fit the tubes into a round metal rack holding approximately 20 tubes and place this rack into the pot. The tubes should be covered with a metal lid, which must not touch the tubes directly. After cooling each tube receives 0.8 ml of chloroform and is well shaken to form an emulsion of sediment and solvent. Now 10 ml of petroleum ether are added, the tubes are tightly stoppered and shaken for several minutes. When two layers have formed the upper petroleum ether layer is removed by suction with a water pump using a capillary with bent tip (see calcium determination). The lower colored layer is again extracted by shaking with 0.4 ml of chloroform and 5 ml of petroleum ether and after separation the upper layer is removed as described above. An additional washing with petroleum ether alone is performed; these washings serve to remove all cholesterol present in the sample. The residue is acidified with the previously determined amount of sulfuric acid (4) and again shaken vigorously for 5 minutes with 2.5 ml of chloroform and 10 ml of petroleum ether. After the two layers have separated exactly 10 ml of the chloroform petroleum ether layer are transferred to an Erlenmeyer flask. It is recommended especially during the summer season to place the tubes in cold water. The solvents are evaporated from a hot plate; the last traces are removed with a water pump (see fig. 50) the flasks being immersed in hot water during this process. Now 5 ml of glacial acetic acid and 4 ml of bromine reagent (5 + 6) are added and 10 minutes later a few crystals of KI and some water (no chlorinated water may be used; if chlorine is present the water must be distilled) are added and the titration performed with  $N/100$  sodium thiosulfate till yellow. Several drops of starch are then added and titration is continued till colorless. For the blank determination it suffices to titrate a mixture of bromine reagent and glacial acetic acid since it has been proved by experience that the reagents used are free from unsaturated fatty acids.

It is recommended to clean all glass ware used in this method with cleaning solution.

Oxalated blood is used for the analysis of whole blood. The oxalated sample must be diluted with an equal volume of distilled water to induce hemolysis. For each determination 0.025 ml of hemolyzed

blood is treated as described above for serum. The dilution factor has to be taken into account when calculating the results.

The following method may be applied for tissues. Approximately 100 mg of material is weighed out cut with fine scissors into small pieces in the indicated amount of water the rest of the procedure being as described above for serum. If it is not desired to remove the cholesterol the extraction in alkaline medium can be omitted.

The calculation is based upon the fact that the value obtained as difference between thiosulfate used up by the blank minus thiosulfate used up by the sample corresponds to the amount of bromine (i.e. iodine) combined with the analytical material. One ml. of N/100 iodine solution contains 1.27 mg. of iodine. Four fifths of the originally measured amount of 0.025 ml. of serum = 0.5 ml. of material was used for the analysis therefore the thiosulfate difference multiplied by 1.27 indicates the amount of iodine used up by 0.5 ml. of serum. If the calculation is to be carried out for 100 ml., a factor of 200 must be applied. The amount in mg. per cent is obtained by multiplication of the thiosulfate difference by 1.27 and by 200.

### *Example*

thiosulfate used up by the blank	7.85 ml
thiosulfate used up by the sample	6.65 ml
	<hr/>
difference	1.20 ml

$1.20 \text{ ml} \times 2.4 = 3.048$  gives iodine combining power of 100 ml. of analytical material.

### *Determination of Iodine Number*

Iodine number is the figure which indicates how many grams of iodine combine with 100 Gm. of fat. The higher this figure runs, the more unsaturated is the compound. In order to determine the iodine number in blood the total fat must be estimated together with the iodine combining power. This can be done in a short time using a relatively small amount of material when working as described under (1). Both determinations can be performed in the extract of 0.025 ml. of serum. The method as mentioned under (1) is followed up to the point where the hydrolyzed material from the Arta pot is extracted.

Instead of 10 ml of chloroform petroleum ether extract (as mentioned before), 6 ml are used for the estimation of iodine combining power. The factor in this case is 423.3 instead of 204 therefore thiosulfate difference multiplied by 423.3 gives iodine combining power in mg per cent. For the determination of total fat 3 ml. of extract is transferred to an Erlenmeyer flask and treated as follows. After careful removal of the solvents first on a hot water bath later with a water pump 10 ml of ceric sulfate reagent are added (151 ml of water and 6 Gm of ceric sulfate are added to 35 ml of N/1 potassium dichromate solution. The suspension is brought into solution by the careful addition of 314 ml of concentrated  $H_2SO_4$ . The oxidation is allowed to take place in an oven at  $120^\circ C$  for 20 minutes. After cooling potassium iodide solution and starch (as indicator) is added and it is titrated with N/40 thiosulfate. As 3 ml. of extract correspond to 0.15 ml of serum the factor 46.08 is applied thiosulfate used up for the blank (L) less thiosulfate used up for the sample (V)  $\times$  46.08 = total fat in mg per cent. The average factor of Blood is used in the calculation 1 ml of N/40 thiosulfate corresponds to 0.06925 mg of lipid. It has to be considered that only  $\frac{1}{3}$  of the material originally measured out has been analyzed. The iodine number (I) of blood results from both values as follows

$$I = \frac{a \times 100}{b} \quad a = \text{amount of iodine taken up by 100 Gm of sample} \quad b = \text{amount of fatty acids present in 100 Gm of sample}$$

#### Example

$$a = 0.228 \quad b = 0.214$$

$$\frac{0.228 \times 100}{0.214} = 106.5 = \text{iodine number}$$

With reference to paragraph (1) the iodine number can be estimated with or without cholesterol included. If cholesterol is considered any change of cholesterol content will affect the calculation by appearing in the numerator as well as in the denominator. However the figures will more or less cancel themselves. As this determination is time consuming the laborious and not absolutely necessary cholesterol extraction may be omitted for practical reasons.

The normal value for unsaturated fatty acids is 170-345 mg per





## Chapter X

# Bile Pigments and Liver-Function Tests

### DETERMINATION OF BILIRUBIN<sup>1</sup>

*Principle of the method* If a diazotized sulfanilic acid (Ehrlich's diazo reagent) is combined with bilirubin a red color results the intensity of which is proportional to the amount of bilirubin present. This coupling reaction of serum may take place either after precipitation of the proteins with 95 per cent alcohol (indirect reaction) or immediately without addition of alcohol (direct reaction) and can be used clinically to distinguish between different forms of jaundice.

#### *Reagents*

(1) diazo mixture a) diazo I 1 Gm. of sulfanilic acid is suspended in a small amount of water it is dissolved by the addition of 15 ml. of fuming HCl and made up to 1 liter with water.

b) diazo II 0.5 per cent aqueous solution of sodium nitrite it will keep only for a short period of time.

Before use 10 ml. of diazo I is mixed with 0.3 ml. of diazo II.

(2) 95 per cent alcohol

(3) standard solution 2.16 Gm. of pure anhydrous cobaltic sulfate (or 3.92 Gm. of cobaltic sulfate  $\times 7 \text{ H}_2\text{O}$ ) is dissolved in water and made up to 100 ml. in a volumetric flask.

#### *Qualitative Determination of Bilirubin (Direct Test)*

One-fourth of a milliliter of serum is placed into each of 2 small test tubes. 0.2 ml. of a freshly prepared diazo-solution is added to one tube and 0.2 ml. of water to the other. If the red color appears within the first thirty seconds it is called a direct prompt reaction. If the color appears only after this time latest within the first five minutes it is called a direct delayed reaction. It is understood that all sera which show a direct reaction—prompt or delayed—will show an indirect reaction immediately after the addition of alcohol. The qualitative test for indirect reaction is performed in the same manner as the quantitative estimation colorimetric comparison and centrifugation being omitted.

*Quantitative Micro- and Semimicro-Determination of Bilirubin**Procedure*

In a small conical test tube 0.4 ml. of 95 per cent alcohol is added to 0.2 ml. of serum; it is well mixed and centrifuged at high speed. With a capillary pipet 0.2 ml. of supernatant is transferred to the cup of a Hellige colorimeter which has been modified by the insertion of a glass wedge (see fig. 53). Then 0.05 ml. of freshly prepared diazo mixture and 0.1 ml. of 95 per cent alcohol are added and after five minutes the colors are compared.

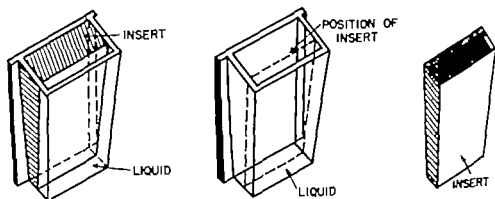


FIG. 53. Insert for cups of Hellige colorimeter permitting work with small volumes of liquid and unchanged thickness of layers.

*Calculation*

The serum has been diluted to 3 times its original volume, or taking into account the contraction, to  $\frac{3}{2}$ . The amount of precipitate formed can be neglected as in all deproteinizing processes. One part of this dilution was again diluted with  $\frac{1}{4}$ th of its volume of diazo reagent and with  $\frac{1}{4}$  of its volume of alcohol. Therefore the final dilution of the original serum is  $\frac{3}{2} \times \frac{1}{4} = 5$ . The color intensity of the cobalt solution corresponds to the intensity of a bilirubin dilution of 1:200,000. This should then correspond to a bilirubin content of  $1:200,000 = 0.5$  mg. in 100 ml. of serum given equal thickness of layers (in the colorimeter) and equal color value. However as shown in the calculation carried out above the serum bilirubin actually

corresponds to 5 times this value. Using a Hellige colorimeter the calculation is done as follows:

Bilirubin content of serum in mg. per cent

$$= \frac{100 - y}{100} \times 0.5 \times 5 = \frac{100 - y}{100} \times 2.5$$

$y$  = colorimeter reading

0.5 mg. of bilirubin in 100 ml. of serum correspond to one bilirubin unit according to Hijnmans van den Bergh.

The following semi-micro method may be applied if more serum is available.

Two ml. of 95 per cent alcohol (2) are added to 1 ml. of serum in a small conical test tube. It is well mixed and centrifuged at high speed. One ml. of clear supernatant is pipetted into the cup of a Hellige colorimeter (see fig. 53). After the addition of 0.25 ml. of freshly prepared diazo-mixture (1) and 0.5 ml. of 95 per cent alcohol five minutes are allowed to pass then the colors are compared. (If the diazo-mixture is too acid the color will occasionally change from red to a bluish purple. In this case a drop of alcoholic ammonia solution is added to produce a red color, similar to that of the standard solution.)

Calculation (see micro-method)

If a serum rich in bilirubin is analyzed it must be diluted with 0.85 per cent NaCl solution before treatment with alcohol. The amount of diluent added being dependent upon the bilirubin concentration of the serum sample. If all available serum has already been used the contents of the colorimeter cup may be diluted with a known amount of dilute alcohol (1 part of 0.85 per cent NaCl + 2 parts of alcohol). The dilution factor must be considered in the calculation.

*Example*

colorimeter reading 45

$$\frac{100 - 45}{100} \times 2.5 = \frac{55 \times 2.5}{100}$$

$$= 1.375 \text{ mg. per cent bilirubin} = 2.75 \text{ units}$$

(see curve, fig. 54)

This method is only suitable for sera giving a negative direct reaction. All other sera will give inaccurate results.

*Qualitative and Quantitative Determination of Direct and Indirect Bilirubin without Deproteinization<sup>1, 2</sup>*

**Principle of the method** A citric acid buffer with added caffeine and urea<sup>4</sup> is used to keep the proteins in solution. Thus the loss of bilirubin adhering to the precipitate is avoided and the "indirect" bilirubin is rendered reactive. The mixture has the additional advantage that the colors are developed at a constant pH, thus eliminating any variations in the colors which may arise because of the indicator nature of the dye substance formed by the diazo reaction.

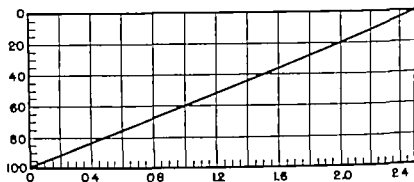


FIG. 54 CURVE FOR BILIRUBIN CALCULATION

*Reagents*

(1) diazo mixture

(a) diazo I One gram of sulfanilic acid is suspended in a little water and dissolved by the addition of 15 ml of fuming HCl. Water is added to 1000 ml.

(b) diazo II A 0.5 per cent solution of sodium nitrite. This solution must be kept on ice. It keeps only a limited time and must be renewed when the diazo dye obtained with bilirubin has a violet tinge. To prepare fresh reagent mix 10 ml of diazo I with 0.6 ml of diazo II.

(2) buffer solution

(a) buffer solution for total bilirubin

15.0 Gm.	citric acid
5.0 Gm.	sodium citrate
	tribasic
5.0 Gm.	pure caffeine
24.0 Gm.	urea (pure)

All above mentioned reagents are dissolved in 60 ml of water in a warm water bath (till  $50^{\circ}\text{C}$ ), cooled and water is added to 100 ml. This solution keeps well.

- (b) buffer solution for direct bilirubin
- |     |  |
|-----|--|
| 24  | Gm urea (pure)   |
| 0.9 | Gm $\text{KH}_2\text{PO}_4$ (potas-<br>sium-monophosphate) |
| 18  | m/15 $\text{H}_3\text{PO}_4$ phos-<br>phoric acid          |

are dissolved and distilled water added up to 100 ml

(3) standard solution 2.16 g purest anhydrous cobalt sulfate (or  $3.92 \text{ g} \times 7 \text{ H}_2\text{O}$ ) are dissolved in water and filled up to 100 ml. in a measuring flask.

(4) normal bilirubin-free serum is diluted 1:5 with reagent 1(a) (one part serum and four parts diazo I)

#### *Procedure*

One-half of a milliliter of pure serum is placed in a test tube. 0.25 ml of freshly prepared diazo-reagent (1) is added and observed to determine whether a color develops within five minutes. Finally 1.75 ml of bilirubin buffer mixture (2a) are added. Depending upon the bilirubin concentration a red color develops either at once or at the latest after fifteen minutes.\*

In the direct reaction (direct bilirubin) the color change occurs after diazotizing only. In the indirect reaction (indirect bilirubin) a red color appears only after the addition of the bilirubin buffer mixture. When the intensity of the color has reached its maximum which as stated takes place at the very latest after fifteen minutes, the material examined is poured into the cup of an Hellige colorimeter and compared with the standard.

Serum especially rich in bilirubin the color intensity of which surpasses that of the standard solution must be diluted either before diazotizing with physiologic salt solution or after diazotizing with buffer solution 2a. The degree of dilution must be considered in the calculation.

When the bilirubin content of the serum is low (below 2.0 mg per cent) there is a difference in the shade of the color in the colorimeter so that at times a comparison of the solutions becomes impos-

sible. This, however, may be remedied in the following manner. The ground glass window at the back of the Hellge colorimeter is removed and the apparatus presented in figure 55 is introduced in its place. This consists of a metal frame at the back of which the ground glass window of the colorimeter is inserted. Into this frame, just

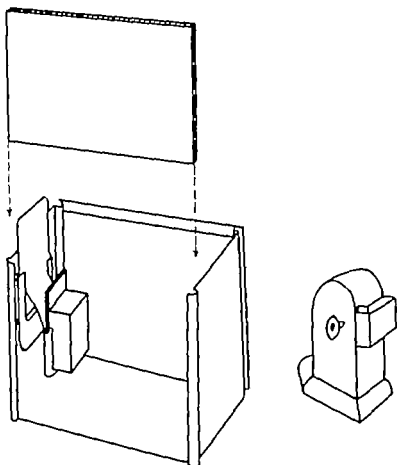


FIG. 55. Insert of Hellge colorimeter for bilirubin determination.

behind the standard another cup is placed. Into this cup the diluted serum as reagent (4) is poured. This solution serving as light filter neutralizes the difference in color between the standard and the unknown.

Even with the use of this filter the shade of color may show variations towards the red or yellow signifying that the serum examined was protein rich (a yellow tinge) or protein poor (a red tinge). In

this case the protein solution filter must be diluted 1:4 or 1:8 depending upon the case.

In general the simple apparatus described above will suffice. If however serial examinations are undertaken it is best to replace the

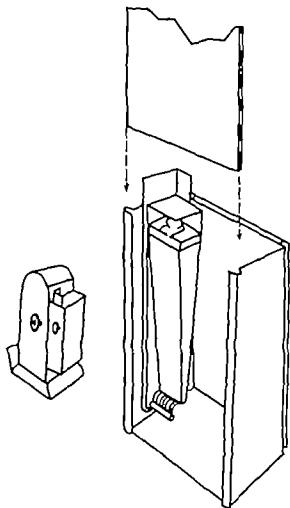


FIG. 56. Glass wedge for bilirubin determination.

chamber for the filter with a moveable hollow glass wedge. Into this a serum diluted 1:3 is placed (fig. 56). By manipulating the filter wedge the color tone of each serum is adjusted and then with the standard wedge the color intensity is determined. The determination may be carried out with less serum even 0.1 ml. if necessary but proportionally smaller quantities of the other reagents must be used.



*Calculation*

$$\text{mg per cent bilirubin} = \frac{(100 - y) \times 2.5}{100}, y \text{ being the number read}$$

off on the colorimeter scale

It may be of interest to determine the direct bilirubin quantitatively in cases where both, direct and indirect bilirubin are present in the same serum

Usually in finding a positive direct reaction, the amount of indirect bilirubin which may be present as well, is neglected and the mg per cent bilirubin given as result includes both kind of bilirubin. To measure the direct bilirubin only, instead of buffer solution 2a after diazotizing the serum, the same amount (1.75 ml) of buffer solution 2b is added. This buffer does not contain any compound with alcoholic groups thus preventing the development of the indirect bilirubin. This buffer solution 2b fulfills the same task of keeping the diazo dye at a constant pH, which enables and simplifies colorimetry. In cases of a direct positive reaction two tests are run one with buffer 2a and one with buffer 2b. The result obtained after subtracting sample 2 from the total amount of bilirubin gives the amount of indirect bilirubin present in the serum.

*Example*

5.7 mg per cent total bilirubin

3.2 mg per cent direct bilirubin

---

2.5 mg per cent indirect bilirubin

High indirect bilirubin values are encountered in cases of hereditary hemolytic jaundice. The color develops very slowly and even after one hour values have been found which seemed too low considering the intense yellow color of the serum analyzed. In these rare occasions it is necessary to select a diazo mixture containing the double amount of  $\text{NaNO}_2$ , i.e. 1.2 ml of diazo II for each 10 ml of diazo I. Using this mixture the indirect color development after addition of buffer is rapid, exact and quantitative.

However, this diazo mixture can not be generally employed, because the difference between direct and indirect bilirubin becomes indistinct. With this diazo mixture direct negative bilirubin will give a direct positive reaction.

In normal serum the bilirubin varies between traces and 0.5 to at

the most 0.75 mg per cent (1-1½ units) 0.5 mg correspond to one bilirubin unit according to H. van den Bergh

A direct prompt reaction together with elevated indirect values is found in all cases of obstructive jaundice. A direct delayed reaction is encountered in early stages of obstructive jaundice in toxic hepatitis catarrhal jaundice acute yellow atrophy of the liver phosphorus—and chloroform poisoning. An increase in bilirubin in the indirect reaction with negative direct bilirubin is noted in hemolytic jaundice.

#### XANTHOCROME SPINAL FLUID

During hemorrhages especially in cases of subdural or subarachnoid hemorrhages the blood pigment is converted to a yellow pigment identical with or closely related to bilirubin giving a positive van den Bergh reaction.

A yellow tint is common in pathologic fluids e.g. in meningitis of all kinds in cerebral or meningeal hemorrhage in cerebral thrombosis in cerebral or spinal tumors, and in polyneuritis. In recent jaundice the fluid is rarely yellow but may become tinged in long-standing cases.

In fresh hemorrhages or in bleeding during spinal taps the spinal fluid appears white after centrifuging and the van den Bergh reaction is negative.

The examination of spinal fluid is carried out as described for serum.

#### LIVER FUNCTION TESTS

The bilirubin content of blood alone does not offer sufficient information about the state of the liver, liver function being of a most complex nature. One liver function test in itself does not give enough indication about the kind and degree of liver damage present. Therefore it seems desirable to perform several liver function tests. Fructose galactose and diospyracetone tolerance tests give certain indications of a disturbance in the carbohydrate metabolism (see pp 262-71). The hippuric acid synthesis test furnishes information about the amino acid metabolism (p. 330) and the Takata Ara test formalin test and to a certain extent the cephalin-cholesterol flocculation test (below) throw some light upon the protein metabolism.

##### (I) *Cephalin-Cholesterol Flocculation Test*

(1) According to Hanger \* \*

(a) Preparation of cephalin

Six hundred grams of fresh sheep's brain are minced by passing

through a meat chopper and dehydrated by letting stand over night with 1200 ml of acetone. This process is repeated 3 times. The brain is filtered with suction and allowed to dry in open air over night whereupon it is powdered and extracted with 300 ml of ether in a Soxhlet. The ether solution is evaporated without filtration to a volume of 100 ml poured into 4 volumes of absolute alcohol and kept on ice for 3-4 hours.

The extract is centrifuged and the residue dissolved in the minimal amount of ether and chilled on ice to precipitate impurities of cerebro-sides. The mixture is again centrifuged the supernatant fluid poured into 4 volumes of absolute alcohol, centrifuged the residue is washed with alcohol and then with acetone and is kept in a desiccator over anhydrous calcium chloride. The yellow substance is ground in a mortar and kept in a test tube over anhydrous  $\text{CaCl}_2$ .

#### (b) Preparation of cephalin-cholesterol emulsion \*

A stock solution is prepared by dissolving 100 mg of sheep's brain cephalin and 300 mg of cholesterol in 8 ml of ether (Squibb's anesthesia). This solution can be kept many months without deterioration in a well-stoppered container. An emulsion of a cephalin-cholesterol complex may be prepared by adding (slowly and with stirring) 1 ml of the stock ether solution to 30 ml of freshly distilled water of 60-70°C and then heating slowly to boiling.

The mixture is allowed to simmer until the final volume is reduced to 30 ml. During the heating, all coarse granular clumps are dispersed to a stable milky translucent emulsion and all traces of ether are driven off. After cooling to room temperature the preparation is ready for testing.

#### *Procedure*

One half of a milliliter of the emulsion is added to a test tube (preferably a centrifuge tube) containing 0.1 ml of the patient's serum diluted with 2 ml of normal (0.85 per cent) saline. After thorough shaking and stoppering with cotton the tube is allowed to stand undisturbed at room temperature and observation is made at the end of twenty-four hours and forty-eight hours as to the amount of flocculation and precipitation that has taken place. With normal human

sera the emulsion remains as a stable homogeneous suspension but with sera from patients with diffuse hepatitis the lipid material tends to flocculate and precipitate to the bottom of the tube.

A ——— reaction indicates a complete precipitation leaving the supernatant liquid water clear. Graduations of the reaction between negative and + + + + are designated in terms of + + + and + + +. No test should be regarded as negative until forty-eight hours have elapsed without flocculation.

Few precautions are necessary. The serum should preferably be fresh or preserved at ice box temperature. Plasma may also be used but the presence of various anticoagulants creates uncontrollable uncertainties. The lipid emulsion if properly prepared remains stable for many days but comparable results can be expected only by measuring accurately the various ingredients and by employing only carefully washed glassware.

Traces of heavy metals or strong acids may give rise to erroneous positive flocculation.

At times the stock solution will furnish a rather labile emulsion. In this case the emulsion must be tested as follows. Three test tubes are filled with 5 ml. each of emulsion 0.5 ml. 1 ml. or 2 ml. of distilled water is added to the different tubes and these various dilutions are added to a series of normal and positive sera. For the analysis the dilution is used which does not cause flocculation of the negative sera after twenty-four or forty-eight hours and which gives a clear cut flocculation in the positive ones.

## (2) According to Krautmann

### Reagents

- (1) alkaline soluble cobaltide (0.5% per cent NaCl solution)
- (2) cephalin chole test addition
- (3) cephalin chole test control unit
- (4) ethyl ether for precipitation

Alkaline soluble cobaltide 1.2 g. and cephalin chole (0.5%) are placed in a 100 ml. volumetric flask and the flask is about 20 ml. of distilled water. The test and control units are added and made up to 100 ml. after the addition of the water. The mixture is placed in the refrigerator for 24 hours before use.

0.55 to 0.6 ml of 0.01 N sulfuric acid using phenolphthalein indicator. The alkalinity should be checked every two weeks).

Preparation of stock cephalin ether solution: exactly 5 ml. of ethyl ether (reagent) are placed into the vial of cephalin-cholesterol antigen as supplied by Difco Co. or 8 ml. of ether to a vial supplied by Wilson & Co. stoppered and shaken well until the antigen dissolves. This stock solution remains stable for a long time at room temperature.

Preparation of cephalin-cholesterol emulsion: 35 ml. of freshly distilled water are placed in a 100 ml. beaker and warmed to about 45° or 50° C. One ml. of the above stock solution is added slowly while stirring with the same pipet. The emulsion is slowly evaporated to a volume of 30 ml. or slightly less on an electric hot plate at about 85–95°C. During evaporation the emulsion is stirred occasionally with a thin glass rod. The beaker is then covered with a watch glass and the emulsion is brought just to the boiling point, and allowed to simmer for 1–2 minutes on the hot plate. The emulsion is poured into a 50 ml. graduated cylinder cooled and if necessary made up to 30 ml. with distilled water.

### *Procedure*

One ml. of the alkaline saline solution is placed in a centrifuge tube. Now 0.02 ml. of blood is drawn from the index finger (as for blood hemoglobin). The finger and lancet must be dry and free from alcohol. For each determination a well washed micropipet\* is used. The tip of the pipet is wiped with absorbent cotton and the blood is blown from the pipet into the tube containing the saline. The pipet is rinsed with the same saline. The blood is immediately mixed by tapping the tube against the palm of the hand (to prevent clotting). The tube is centrifuged and the supernatant is drained into another centrifuge tube where exactly 0.1 ml. of working cephalin-cholesterol emulsion is added and mixed by tapping the tube as above described. The tube is then stoppered with a rubber stopper. The contents should not be mixed by inverting the tube. Two controls with known positive and negative sera are prepared (the sera are kept for this purpose in the refrigerator).

Readings are made in twenty four and forty-eight hours against a

\* It is recommended to use a pipet fashioned from a thermometer capillary. It serves as a lens and facilitates reading.

black background as follows Negative (-) no flocculation Plus minus (+-), very slight flocculation. One plus (+) slight flocculation with turbid supernatant fluid Two plus (++), flocculation with slight turbidity of liquid. Two plus minus (++-), flocculation as above but with slightly greater turbidity Three plus (+++), heavy flocculation with very slight turbidity of fluid Four plus (++++), heavy and complete flocculation with water-clear supernatant fluid

If serum is used for the analysis 0.02 ml. of serum and 0.1 ml. of cephalin is added to 1 ml. of alkaline saline solution. Centrifugation is omitted and the remainder of the procedure and reading is performed as described for whole blood

### *(II) Formalin Flocculation Test\**

This test was originally described by Akashi for malaria. However, in checking these experiments it could be demonstrated that the reaction was positive only in such cases of malaria where the reticulo-endothelial system had been disturbed (enlarged liver and spleen). In uncomplicated malaria cases the test was generally negative. A positive reaction was also found in cirrhosis of the liver and in severe cases of jaundice. Therefore, the positive reaction indicates liver damage, as soon as the liver returns to a normal state of health the reaction becomes negative. With few exceptions the results of this test run parallel with the results of the cephalin test.

#### *Reagents*

*Three-tenths per cent formalin solution* This solution must be freshly prepared for each test by diluting 0.3 ml. of 40 per cent formalin to 100 ml. with redistilled water.

#### *Procedure*

One ml. of formalin solution is added to 0.2 ml. of serum. The mixture is shaken and allowed to stand at room temperature for two hours and centrifuged. A flaky appearance on examination with a lens indicates a positive reaction. A strong reaction is seen with the naked eye after a few minutes.

*(III) Hippuric Acid Synthesis Test\* (Micro-Method)*

Quick has observed that the hourly rate of excretion of hippuric acid in normal persons following the ingestion of sodium benzoate is remarkably constant while in certain types of disease of the liver it is markedly reduced. This reaction is due primarily to the diminished capacity of the liver to synthesize amino-acetic acid and in part to damage of the enzymatic mechanism which unites benzoic acid with amino-acetic acid. The output of hippuric acid after the ingestion of benzoic acid is considered a measure of the liver's capacity to furnish amino-acetic acid and an index of its detoxifying power.

*Procedure*

One hour after a light breakfast of coffee and toast the patient is given 6 Gm. of sodium benzoate dissolved in 30 ml. of water, preferably flavored with oil of peppermint. This is followed by one-half glass of water. Immediately after taking the drug the patient voids, and then he collects complete specimens of urine hourly for four hours. Should the analysis be delayed more than ten hours the samples are preserved with toluene.

*Reagents*

- (1) 0.1 per cent alcoholic tropaeoline solution or congo paper
- (2) NaCl c.p.
- (3) 18 per cent HCl (one part of conc. HCl and one part of distilled water)
- (4) 30 per cent NaCl solution saturated with hippuric acid (A few crystals of hippuric acid are added to a saturated NaCl solution it is boiled for a very short time, cooled with ice and filtered)\*
- (5) 1 per cent alcoholic phenolphthalein solution
- (6) 1 per cent alcoholic dimethylamino-azobenzene
- (7) N/4 NaOH
- (8) 30 per cent zinc sulfate solution
- (9) N/1 NaOH

*Procedure*

One and five tenths grams of NaCl and 5 ml. of urine taken from the four hour collection are added to 2 round bottom centrifuge tubes

\* If no hippuric acid is available the hippuric acid isolated from urine after sodium benzoate administration may be recrystallized and used.

the salt is dissolved by stirring carefully with a thin glass rod. Then 2-3 drops of  $\frac{1}{2}$  concentrated HCl (3) is added until the mixture reacts acid to congo paper or tropeolin solution and the precipitation of hippuric acid is induced by rubbing the walls of the tube with a glass rod.

After standing in ice water for fifteen minutes the tubes are centrifuged for ten minutes at high speed and the supernatant is removed using a water pump and the apparatus as shown in figure 24. One ml. of ice cold 30 per cent NaCl solution, saturated with hippuric acid is added to the sediment well shaken and the walls of the tube are rinsed with another 2 ml. of this solution. After centrifuging the procedure is repeated 2 or 3 times until the acid reaction has disappeared. The residue is dissolved in hot distilled water transferred quantitatively to a Hagedorn-Jensen tube and titrated while hot with N/4 NaOH, using phenolphthalein and dimethylamino-azobenzene as indicator, until the color changes from orange through yellow to red.

Large amounts of protein or bilirubin possibly present in the urine must be removed as follows. 0.5 ml. of 30 per cent  $\text{ZnSO}_4$  solution and 0.025 ml. of N/1 NaOH are added to 10 ml. of urine and filtered. 0.50 ml. of filtrate is treated as described above.

#### Calculation

To the amount of sodium benzoate (as hippuric acid) determined by titration the amount remaining dissolved in the urine after precipitation is added. This is calculated on the basis of 0.10 Gm. per 100 ml. at room temperature. Where  $\lambda$  is the number of milliliters of N/4 NaOH used in titration the total amount for the specimen in terms of sodium benzoate is as follows:

$$\lambda \times \frac{\text{vol. of specimen}}{5} \times 0.036 + \left( 0.10 \times \frac{\text{vol. of specimen}}{100} \right) \\ = \text{Gm. sodium benzoate excreted}$$

The factor 0.036 was determined as follows. A normal sodium benzoate solution ( $\text{C}_6\text{H}_5\text{COONa}$ ) corresponds to 144 Gm. one liter of a N/4 solution contains 36 Gm. therefore 1 ml. of a N/4 NaOH corresponds to 0.036 Gm. of sodium benzoate.



*Example*

Volume of urine = 250 ml

N/4 NaOH used up for the titration of 5 ml of urine 2.3 ml

$$2.3 \times \frac{250}{5} \times 0.036 + \frac{0.10 \times 250}{100} = 4.14 + 0.25 = 4.39 \text{ Gm.}$$

4.39 Gm of sodium benzoate were excreted

According to this method the normal adult excretes about 3-5 Gm. of benzoic acid in the form of hippuric acid in four hours. Values below 2.4 Gm (= 40 per cent) are considered pathologic.

Diminished output occurs in catarrhal jaundice, intrahepatic obstructive jaundice, acute and subacute hepatic necrosis, cancer or syphilis of the liver after operation of the biliary tract, in advanced cardiac decompensation with passive congestion of the liver. The test is also positive in advanced kidney diseases, in some cases of hyperparathyroidism, cachectic states and in anemia. Usually the test is normal in uncomplicated diseases of liver and bile ducts.

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## Chapter XI

# Enzymes

### DETERMINATION OF DIASTASE (AMYLASE) IN SERUM

*Principle of the method* Equal amounts of a known starch solution are added to tubes containing serial dilutions of the serum to be analyzed. After incubation at  $38^{\circ}\text{C}$  for 30 minutes the lowest serum concentration is noted which is still able to ferment the starch.

#### *Reagents*

(1) 0.1 per cent starch solution 0.1 Gm. of soluble starch (weighed on an analytical balance) is stirred up with a little cold distilled water and 80 ml. of boiling distilled water is then added with shaking. After heating in a boiling water bath for several minutes, and subsequent cooling the volume is made up with water to 100 ml. in a volumetric flask. If  $\text{HgI}_2$  (rods or plates, see append. p. 371) is added as preservative the solution will keep indefinitely when stored on ice.

(2) N/50 iodine-potassium iodide solution

(3) 0.85 per cent  $\text{NaCl}$  solution

#### *Procedure*

Two tenths (0.2) ml. of 0.85 per cent  $\text{NaCl}$  is added to each of 10 small test tubes (length 65 mm. diameter 16 mm.). 0.2 ml. of serum is added to the first tube and after mixing with a pipet by drawing up and blowing out the mixture 0.2 ml. of this dilution is transferred to the second tube. Here it is mixed thoroughly as described above and 0.2 ml. of this dilution is transferred to tube Nr. 3. This process is repeated until all ten tubes contain increasingly diluted serum. The 0.2 ml. removed from the last tube is discarded. In this manner a dilution series of  $2^{-1}$  to  $2^{-10}$  is obtained. Using an exactly calibrated capillary pipet 0.4 ml. of starch solution (1) is now added to each tube and after thorough mixing the samples are placed in a water bath of  $38^{\circ}\text{C}$  for thirty minutes. They are cooled rapidly and 1 drop of iodine solution (2) is added to each tube. The first tube

showing traces of a purple color indicates the borderline up to which the diastase was active

### Calculation

One diastase unit (D U) represents the amount of enzyme (serum dilution) which ferments 0.2 ml of a 0.1 per cent starch solution under the above mentioned conditions. Since 0.4 ml of 0.1 per cent starch solution is used in this method, 2 diastase units are determined in each analysis.

The amount of diastase present in the sample is obtained according to the equation  $D 38^{\circ}/30 \text{ min} = 2^{x+1}$ ,  $x$  representing the tube where the entire amount of added starch has been fermented and where no purple color is visible after the addition of iodine.

### Example

A purple color appears in tube 6 after iodine addition. Therefore, the diastase was active only up to tube 5 (tubes 1-5 colorless after iodine addition, tubes 6-10 colored after iodine addition). The amount of diastase is  $= D 38^{\circ}/30 \text{ min} = 2^{x+1} = 64 \text{ units}$ .

Under normal conditions the diastase is excreted in the urine. The blood diastase may be increased under pathologic conditions. Normal value 32-64 diastase units. Values up to 2000 units and more have been reported under pathologic conditions. The diastase (amylase) is increased in acute pancreatitis, cancer pancreatitis, some cases of perforated ulcer, severe kidney disease. Low values are found in hepatitis with necrosis, severe burns, some cases of pneumonia, congestive heart failure.

## DETERMINATION OF PHOSPHATASE<sup>1</sup>

### Determination of Alkaline Phosphatase

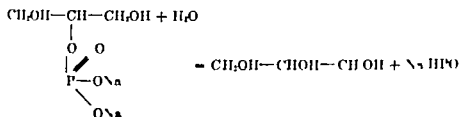
**Principle of the method** Phosphatase splits glycerol phosphoric acid into glycerol and free phosphoric acid. The amount of phosphoric acid liberated is an indication of the enzyme concentration in the analysed serum.

### Reagents

(1)  $\beta$ -glycero-phosphate solution<sup>2</sup>: 2.5 Gm of  $\beta$ -glycero-phosphate and 2.12 Gm of monosodium-diethylbarbituric acid are dissolved in

about 20 ml. of water in a 50 ml. volumetric flask. To this 20 ml. of pure glycerin\* is added and the flask is then filled up with water. This stock solution has to be diluted 10 times before use, i.e. one part of concentrated reagent is added to 9 parts of water. The stock solution keeps indefinitely on ice; the dilution only a short time.

The stabilization is obtained by adding glycerin, which prevents bacterial decomposition as well as hydrolysis according to the law of mass action. β-glycerophosphate is decomposed by hydrolysis into glycerine and phosphoric acid as follows:



By means of an excess of glycerine the formation of free phosphoric acid is prevented, i.e. the equilibrium constant is changed in favor of the ester formation.

According to G. J. Shinowara<sup>1</sup> the optimum activity of alkaline phosphatase is at  $\text{pH } 9.30 \pm 0.15$ . The simplest way of adjusting the substrate to the desired pH is as follows:

To 1.8 ml. of secondary m/15 phosphate buffer solution ( $\text{pH} = 9.18$ , see p. 351) in a test tube are added 3 drops of 0.01 per cent aqueous thymol blue solution. In a second similar tube 3 drops of 0.01 per cent aqueous thymol blue solution are added to 0.5 ml. of concentrated substrate and 1.5 ml. of water. To this latter tube  $\text{N } 10 \text{ NaOH}$  is added from a pipet with bent tip (see fig. 1) until the colors in both tubes are identical. The entire amount of substrate is now diluted with an amount of  $2 \text{ N NaOH}$  corresponding to the amount of alkali used in the test titration.

(2) Fourteen per cent trichloroacetic acid solution

(3) Reagents as described for phosphorus determination (see micro method for the determination of free phosphoric acid (p. 413))

#### Procedure

Into 2 short round bottom test tubes equipped with ground glass stoppers 1 ml. of sodium glycerophosphate each is placed. From a volumetric pipet bearing 2 marks (see alcohol determination) 0.1 ml.

of serum is delivered slowly into each tube (blowing might alter pH) and well mixed. The serum should not be kept for more than six hours before starting the analysis, as changes in the enzyme occur after that time. One tube is immersed in a water bath at  $38^{\circ}\text{C}$  for exactly one hour. To the other tube 0.9 ml of trichloroacetic acid (2) is added and the tube is centrifuged at high speed. After one hour, tube no. 1 is removed from the water bath and rapidly in ice water, 0.9 ml of trichloroacetic acid (2) is added and the tube is centrifuged at high speed. 1.5 ml of each clear supernatant is transferred to a dry test tube and the phosphorus is determined (see p. 93). \*

### Calculation

One phosphatase unit is defined as the amount of enzyme which liberates 1 mg per cent of phosphorus from the buffered glycerol phosphoric acid substrate after one hour's incubation at  $38^{\circ}\text{C}$ . If higher phosphatase values are expected, the incubation time may be shortened; if less enzyme is expected, the incubation time may be prolonged and the following factor included in the calculation.

Incubation time (in minutes)	10	15	20	30	60	120	180	240
factor	4.70	3.30	2.57	1.82	1.00	0.55	0.39	0.28

### Example

before incubation	4.2 mg per cent phosphorus
after incubation (60 min.)	10.5 mg per cent phosphorus
difference	6.3 mg per cent phosphorus

the phosphatase content is 6.3 units. If the incubation time is shorter the following calculation is performed:

before incubation	4.2 mg per cent phosphorus
after incubation (30 min.)	7.6 mg per cent phosphorus
difference	$3.4 \times 1.82 = 6.18$ units

The normal alkaline phosphatase values vary between 1.5-4 u. Higher levels may be found in jaundice, generalized osteoporosis,

When calculating the phosphorus content, the value obtained must be multiplied by 2, as half the amount of serum has been analyzed.

(5-10 units) hyperparathyroidism (approx. 20 units) Paget's disease (35-50 units) Low values are found in chronic nephritis with destruction of renal tissue and in celiac disease Normal values for children range between 5 and 12 units higher values are found in acute rickets (30-100 units) healed rickets are accompanied by values of 6-14 units

### *Determination of Acid Phosphatase*

E. B. Gutman and A. B. Gutman<sup>4</sup> found that the phosphatase activity in *acid* medium is elevated in diseases of the prostate The authors used acid phenyl phosphate ( $\text{pH} = 4.9$ ) as substrate and estimated the liberated phenol However it is simpler to use glycerophosphate as substrate which is adjusted to  $\text{pH } 5.0 \pm 0.15$  as follows

The following solutions are measured out into a test tube 2 ml of phosphate solution ( $= 0.1 \text{ ml of } \text{Na}_2\text{HPO}_4 + 4.9 \text{ ml of } \text{KH}_2\text{PO}_4$ ,  $\text{pH } 5$  see p 384) 1 ml of indicator ( $= 0.025$  per cent aqueous  $\gamma$ -dinitrophenol solution) and 4 ml of 0.9 per cent  $\text{NaCl}$

Into a second tube of the same diameter are placed 0.1 ml of serum 5.9 ml of water 1 ml of dilute substrate (1:10) and 1 ml of indicator

Using a bent pipet (fig 4)  $\text{N}/1 \text{ HCl}$  is added to the second tube until the color in both tubes is identical The total amount of concentrated substrate is now diluted with an amount of  $5 \text{ N HCl}$  corresponding to the above titration

Acid and alkaline phosphatase may be determined together by using either the acid or the alkaline substrate the procedure and calculation in both cases is the same

Normal value for acid phosphatase	0.2-1.1 units
diseases of the prostate	1.2-31.7 units and higher

### DETERMINATION OF LIPASES

#### *Titrimetric Determination of Lipase<sup>5, 6</sup>*

*Principle of the method* The substrate used in the test is an emulsion of olive oil The degree of lipase activity is indicated by the amount of fatty acid liberated and it is reported in terms of ml of  $\text{N}/20$  sodium hydroxide solution

#### *Reagents*

(1) emulsion of olive oil This should be purchased from a com

mercial laboratory It is prepared in a homogenizer from equal parts of pure olive oil free from fatty acid, and a 5 per cent solution of gum acacia, with 0.2 per cent of sodium benzoate added as a preservative

(2) phosphate buffer solution pH 7 Prepare Soerensen phosphate buffer solution from a m/15 solution of primary potassium phosphate and m/15 secondary phosphate solution, adjusted to pH 7 (see p 386)

(3) N/20 NaOH solution

(4) 1 per cent solution of phenolphthalein in alcohol

(5) 95 per cent ethyl alcohol

### *Procedure*

One ml of serum is placed in a test tube 2 ml of olive oil (1) 3 ml of dist water and 0.5 ml of buffer solution (2) are added The mixture is shaken and incubated at 38°C for twenty four hours A blank is prepared by placing 1 ml of serum in a test tube adding 3 ml of distilled water and heating at 70°C for about five minutes to destroy the enzyme This is followed by adding the buffer and the substrate shaking the mixture and incubating it at 38°C for twenty four hours Now 3 ml of alcohol and 2 drops of phenolphthalein (4) are added to both tubes and the mixtures titrated to a permanent pink color of the indicator with N/20 NaOH The number of ml of NaOH used to neutralize the blank (tube 2) subtracted from the number of ml of NaOH used to neutralize the acidity in tube 1 is recorded as ml of N/20 NaOH for each ml of serum

### *Interpretation*

According to Comfort the normal range is from 0.2 ml to 1.5 ml of N/20 NaOH for each milliliter of serum A sharp rise even up to 9-10 ml may occur in acute pancreatitis and other injuries to the gland Further high values may be found in cases of cholelithiasis with jaundice, cirrhosis liver—and pancreas carcinoma

### *Determination of Lipase Resistant to Quinine*

The surface tension of a saturated solution of tributyrin in water is much lower than that of water On addition of serum, which contains lipase the ester hydrolyzes into glycerin and butyric acid and the surface tension of the solution gradually increases until upon com-

pletion of enzymatic hydrolysis it reaches the value of water. The changes in the surface tension are measured by the stalagmometric method.

The lipase is normally poisoned by quinine, atoxyl and similar drugs and in their presence the serum loses its saponifying property. However lipases appear in certain liver and kidney injuries which are resistant to these poisons and hydrolyze fats in spite of their presence.

### *Reagents*

(1) buffer solution 1.27 Gm. of citric acid ( $C_6H_8O_7 \times 1 H_2O$ ) and 33.35 Gm. of  $Na_2HPO_4 \times 2 H_2O$  are dissolved in water and made up to 1000 ml.

(2) Saturated solution of tributyrin 1-3 drops of tributyrin are added to 100 ml. of water vigorously shaken for 20 minutes and filtered through a folded filter.

(3) Before use 50 ml. of saturated tributyrin solution (2) are mixed with 3 ml. of buffer solution (1).

(4) Quinine solution 0.5 Gm. of quinine hydrochloride are dissolved in 100 ml. of water.

### *Apparatus*

The Rona stalagmometer (fig. 57) should possess the volume of 3 ml. the number of drops of distilled water at 20°C. delivered by the apparatus should be 95 ( $\pm 5$ ). Between determinations the stalagmometer is left in potassium dichromate-sulfuric acid solution. Before use it is washed thoroughly with distilled water then with the solution being determined. The apparatus must not be washed with organic solvents like alcohol, acetone, ether etc. The tip should not be touched by fingers and should therefore be protected by keeping it inside a test tube. For the test the apparatus is placed in an exactly perpendicular position and filled by means of suction through rubber tubing attached to the upper end. The stalagmometer should be kept in a place well protected from water vapors and humidity. The solution to be analyzed should be kept at a constant temperature of 20°C. and the determination performed in a room where the temperature does not rise above 20°C.



*Procedure*

Six tenths (0.6) of a milliliter of fresh serum is mixed in a test tube with 0.2 ml of quinine solution (4), the tube is stoppered and kept for thirty minutes at  $20^{\circ}\text{C}$ . Then 10 ml of buffered tributyrin solution (3) are added to the tube, the contents well mixed and used first to

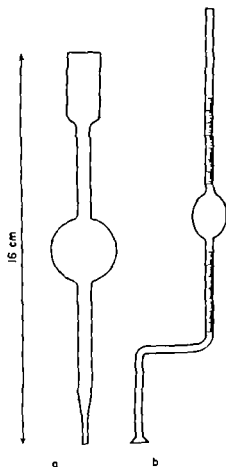


FIG 57 Stalagmometer a According to Rona b According to Traube

wash and then to fill the stalagmometer. After three minutes the number of drops of the stalagmometer are counted, the count repeated and the results noted.

Another count is made after exactly ninety minutes. From the difference between the immediate and later count the amount of quinine resistant lipase can be estimated.

*Evaluation of the results*

Difference in number of drops less than five is regarded as a negative result. If the difference is larger than five the result is positive.

From 6 to 10 drops	suspicious (+)
From 10 to 15 drops	slightly positive (++)
above 15 drops	strongly positive (+++)

The presence of quinine-resistant lipase points to clear injury of the liver. Thus lipase appears sometimes in extra hepatic morbid changes. A severe icterus accompanied by suspicious (+) lipase content suggests an obstruction icterus, whereas the result (++) and (+++) indicate quite clearly an injury of the liver parenchyma.

In acute hepatitis the reaction is usually positive and its degree changes with the progress of the disease.

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## Chapter XII

# Vitamins

### METHOD FOR DETERMINATION OF ASCORBIC ACID CONTENT OF BLOOD<sup>1</sup> (VITAMIN C)

*Principle of the method* Ascorbic acid reduces dichlorophenolindophenol sodium. The amount of color reduction of a dichlorophenolindophenol sodium solution acted on by plasma filtrate is measured and calculated in terms of ascorbic acid.

#### *Reagents*

- (1) finely powdered sodium or lithium oxalate
- (2) 10 per cent sodium tungstate solution
- (3) 2.3 N  $\text{H}_2\text{SO}_4$  (9.076 Gm per cent of  $\text{KHSO}_4$  may be used instead)

(4) dye solution Approximately 0.1 Gm of dichlorophenolindophenol sodium (E. K. Co. Nr. P 3463) is twice extracted with scalding hot water (for maximum solution). Each extract is poured through a small filter paper combined and diluted with freshly boiled and cooled water to a volume of 50 ml. The insoluble residue is rejected. This stock solution is good for about three weeks kept on ice. Ten milliliters of this stock solution is diluted to 100 ml with boiled and cooled distilled water. This diluted solution after standardization is used for titration of the deproteinized plasma fluid.

#### *Standardization of the dye solution*

Approximately 100 mg of ascorbic acid (from a sealed ampule) dissolved in 100 ml of 5 per cent acetic acid in a volumetric flask. Five milliliters of this solution are titrated immediately with N/10 iodine solution. One milliliter of N/100 iodine solution corresponds to 0.877 mg of ascorbic acid. The iodine solution has to be prepared fresh for each determination (it is not stable). 3.567 Gm of  $\text{KIO}_3$  (free of water) are dissolved in about 500 ml of water in a 1000 ml volumetric flask. 10 ml of N.1  $\text{H}_2\text{SO}_4$  are added and the volume made up to the mark with water. This solution can be kept indefinitely provided it is protected from sunlight and held in a flask with ground stopper.

Two milliliters of this solution are treated with 0.5 Gm of KI and made up with water to 100 ml (N/100 iodine solution)

If 2 ml of the ascorbic acid solution (approximately 100 mg per cent) are oxidized by (e.g.) 2.1 ml of N/100 iodine solution then 2 ml of ascorbic acid solution contain  $2.1 \times 0.877 = 2.1018$  mg of ascorbic acid. One milliliter of this solution is made up to 200 ml with 5 per cent acetic acid in a 200 ml volumetric flask, 2 ml of this dilution containing 0.010,21 mg of ascorbic acid are titrated with the diluted dye solution (4) until a persistent red color appears. Two milliliters of ascorbic acid solution requires (e.g.) 1.57 ml of dye solution, whereas 2 ml of 5 per cent acetic acid alone require 0.06 ml till the appearance of a persistent red color, therefore 0.010,21 mg of ascorbic acid require  $1.57 - 0.06 = 1.51$  ml of dye i.e. 0.01 mg of ascorbic acid correspond to 1.13 ml of dye solution.

### Procedure

Five or more milliliters of blood are drawn and oxalated. The blood is immediately centrifuged, the plasma removed, and a tungstic acid filtrate prepared. Usually 2 ml of plasma are pipetted into a round bottom 15 ml centrifuge tube, 6 ml of distilled water added, followed by 1 ml of 10 per cent sodium tungstate, the contents mixed, then 1 ml of 2/3 N sulfuric acid added. After thorough mixing, the tube is allowed to stand for 1-2 minutes then centrifuged. About 6 to 7 ml of clear supernatant is obtained. Two ml portions are pipetted into round bottom centrifuge tubes and titrated immediately to the first faint pink color (compared with the untitrated solution) as rapidly as possible. A 3 ml micro-burette (with 0.01 ml divisions) is used to measure the required volume of dye solution.

A blank determination is carried out in the same manner by titration of 2 ml of the following mixture: 8 ml of water, 1 ml of 10 per cent sodium tungstate and 1 ml of 2/3 N  $\text{H}_2\text{SO}_4$ .

### Example

Two ml of tungstic acid filtrate representing 0.4 ml of plasma require 0.47 ml of dilute dye solution, till the appearance of the color the blank requires similarly 0.06 ml. Therefore 0.4 ml of plasma require  $0.47 - 0.06 = 0.41$  ml of dye solution or 1 ml of plasma requires  $2.5 \times 0.41 = 1.025$  ml.

$$\text{According to the equation } \frac{0.01}{X} = \frac{1.43}{1.025}$$

$$X = 0.00716$$

The ascorbic acid content of the plasma is 0.716 mg per cent. The calculation can be performed according to the general formula

$$\frac{\text{ml. of dye solution used for titration} \times 2.5 \times 100}{\text{ml. of dye solution corresponding to 0.01 mg. ascorbic acid}} = \text{mg. per cent ascorbic acid}$$

The normal level of ascorbic acid in blood ranges between 0.7-1.4 mg per cent, values below 0.7 mg per cent indicate a deficiency of the vitamin.

### PROTHROMBIN DETERMINATION

The prothrombin concentration test is used as an aid in the diagnosis of diseases of the liver and as a guide in the vitamin K—and dicumarol therapy.

*Principle of the method* The Quick prothrombin test<sup>2, 3, 4, 5, 6</sup> is an indirect measurement of the plasma prothrombin concentration. It is based on the theory of blood coagulation proposing that thromboplastin in the presence of calcium converts prothrombin to thrombin, which in turn converts fibrinogen to fibrin, the strands of which are the end results of clotting. With rare exceptions the variations in the concentration of calcium and fibrinogen encountered in man do not affect the blood coagulation time. Therefore the speed of the reaction after addition of an excess of thromboplastin is used as a measure of the amount of prothrombin present. The standard of reference is the time required for the reaction to take place in the normal subject (= 100 per cent).

#### Reagents

- (1) 0.025 M calcium chloride
- (2) 0.1 M sodium oxalate
- (3) barium sulfate used for X ray, 30 per cent suspension in water
- (4) thromboplastin
- (a) preparation and storage of dehydrated brain

Fresh rabbit brain or preferably human brain obtained at autopsy, is stripped off its pia and macerated under acetone in a mortar. The acetone is replaced as many times as necessary to obtain a granular nonadhesive powder which is dried on a water suction filter (or on air). The completely dry powder is kept on ice under vacuum either in vials\* or in a calcium chloride desiccator evacuated by means of an oil suction pump. After each removal of material required for testing the vacuum is renewed. Thus the dehydrated brain keeps unchanged for a very long period.

(b) preparation of thromboplastin

0.15 Gm. of dehydrated brain is mixed with 12.25 ml. of fresh saline and 0.25 ml. of sodium oxalate (2) is added. The mixture is extracted in a water bath at 50°C. for fifteen minutes, mixing frequently. The suspension is placed for several hours at room temperature to allow the large particles to settle on the bottom. The supernatant suspension is separated from the sediment, mixed well and placed in test tubes in 2.5 ml. to 5 ml. amounts (according to daily request) and tightly stoppered. The lot is stored in a frozen state in a freezing unit. Before use this frozen extract is thawed at 37°C. for thirty minutes, agitating the tube occasionally while thawing.

The frozen thromboplastin extract retains its potency for at least three months. So if storage in a freezing unit is possible the amount required during about three months can be prepared at one time. This method secures a fairly large supply of thromboplastin of equal potency whilst daily weighing out of small amounts of dehydrated brain cannot produce homogeneous extracts.

The amount of saline added to 0.15 Gm. of dehydrated brain depends on its potency. Generally the dehydrated brain gives good results when diluted in the above proportion.

When a new batch of dehydrated brain gives weaker or more potent thromboplastin extracts it can usually be concentrated respectively

Instead of storing the thromboplastin in a vial it may also be kept in a bottle with screw top (catsup bottle). Several holes should be drilled through the top. The inside of the top is smoothed out and covered with a round piece of rubber. A hypodermic needle is inserted through the rubber and the needle connected to a vacuum pump. After vacuum is established the needle is removed.

To take out thromboplastin for a test the lid is unscrewed and the rubber plate removed. Later on the top is put back on the bottle and the bottle again evacuated.

diluted by using a smaller or larger amount than 12.25 ml. of saline for the extract of 0.15 Gm. of brain, in order to obtain the clotting times corresponding to the standardization curve.

(c) Standardization of thromboplastin

The thromboplastin is standardized by doing prothrombin time determinations on several dilutions of plasma of several normal persons, using barium sulfate treated plasma as diluent.

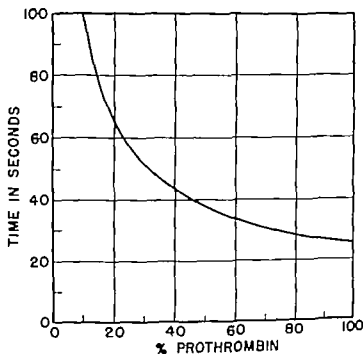


Fig. 58 Curve for prothrombin calculation

As variations in prothrombin concentrations between 100 per cent and 30 per cent produce little change in clotting time, an exact curve for the prothrombin concentration in per cent of mean normal is difficult to obtain. When the plasma is diluted to 10 per cent, however, very slight changes in prothrombin concentration result in large changes in the clotting time. So we developed a curve (fig. 58) based on 10 times diluted plasma only.

To 10 ml. of normal plasma in a centrifuge tube 2 ml. of 30 per cent barium sulfate suspension is added, shaken well, incubated at 37°C for ten minutes and then centrifuged at 3000 RPM for ten minutes. The supernatant fluid is prothrombin free plasma.

The normal plasma is ten times diluted with the barium-sulfate treated plasma. 4.5 ml. of barium sulfate-treated plasma is added to 0.5 ml. of normal plasma. This 10 per cent dilution represents 100 per cent prothrombin.

Further dilutions are prepared in the following way:

Barium Sulfate Treated Plasma		Plasma 10 Times Diluted	% Prothrombin
0.45	+	0.05	10
0.40	+	0.10	20
0.25	+	0.25	50
0.15	+	0.35	70
0.05	+	0.45	90

This is repeated with several normal plasma samples, the average results producing the curve shown in figure 58.

During some time patients' plasma after vitamin K or dicumarol treatment are examined undiluted and in 10 per cent dilutions with the identical barium sulfate treated plasma. The prothrombin in per cent of normal of the 10 times diluted plasma is read on the above curve. The clotting time of the whole plasma is reported. After experience of some weeks the time consuming dilutions can be omitted and the reported results of whole plasma can produce the table (fig. 58a) for prothrombin concentration of undiluted plasma, based on the 10 per cent dilution curve. The results thus obtained are sufficient for clinical use although occasionally the 10 per cent dilution can be performed when special precision is wanted.

### *Procedure*

From each patient, 1.8 ml. of venous blood are drawn into test tubes containing 0.2 ml. of sodium oxalate. It is convenient to use test tubes with a mark at 2 ml. so that the blood need not be measured exactly in the syringe. The blood is mixed by gentle inversion and kept on ice till ready for examination. When all samples are taken, they are centrifuged at about 2000 RPM for five minutes and placed on ice again. With a 1 ml. serologic pipet (bent at the tip) 0.1 ml. of plasma from one sample (not more than 6 samples at one time) is transferred into three test tubes each. All 0.1 ml. portions of



plasma are kept on ice till actually ready to begin testing. Plasma which is removed from the red blood cells diminishes its prothrombin content after a very short time, even when kept on ice, whereas plasma which remains together with the erythrocytes keeps its prothrombin content unchanged for more than six hours when kept on ice. In a water bath of 37–40°C are kept, in one test tube, 0.025 M calcium chloride solution with a dropping pipet (2 drops calcium chloride being 0.1 ml) and in a second test tube thromboplastin with a 1 ml serologic pipet inside.

Clotting Time in Seconds	Per cent Prothrombin
15	100
15.5	90
16	80
16.5	70
17	60
18	50
19	45
20	40
21	35
22	30
23	25
24	20
25	15
26	10
27	less than 10

FIG. 58a.—Table for prothrombin concentration of undiluted plasma

One test tube containing 0.1 ml of plasma is placed into the water bath for thirty seconds, 0.1 ml of warmed thromboplastin is added and the contents are gently mixed. Finally 2 drops of calcium chloride are added and timing is started with a stop watch when the first drop reaches the fluid. The contents are mixed immediately. After ten seconds the tube is gently tilted in and out of the water bath until a clot is about to form. The time is noted from the stop watch. The clotting times of three such 0.1 ml portions are averaged as the prothrombin time of whole plasma. The per cent prothrombin corresponding to the observed prothrombin time are read from the chart (fig. 58).

*Example*

Full plasma clots after 23 seconds

Ten timed diluted plasma clots after 50 seconds

Reading the curve 25 per cent is found i.e., 23 seconds equals 25 per cent

## MICRO-DETERMINATION

(a) *In plasma*

In cases when venipuncture is difficult or in small children the micromethod can be applied. Glass tubes as for the determination of the sedimentation rate (see p. 11) are used for this purpose. 0.36 ml. of sodium oxalate (2) is measured with this pipet into a small test tube and the remainder of the oxalate solution discarded. Now blood is drawn from the finger tip up to mark 0 and blown into the test tube containing the sodium oxalate. Rinse the pipet with separately kept sodium oxalate once, draw blood up to mark 0 again and add it to the first portion. The blood is now diluted 1:10 with the sodium oxalate (3.1 ml. blood + 0.36 ml.<sup>3</sup> sodium oxalate). The plasma thus obtained suffices for 2 or 3 controls of prothrombin time estimation. If even this amount of blood is not easily obtainable 0.02 ml. of oxalate can be mixed with 0.18 ml. of blood using a 0.1 ml. serologic pipet. This plasma can be used for a 1:10 solution with barium plasma taken from another blood sample.

(b) *Bedside test in blood*

For the determination of prothrombin in infants very small amounts of blood are required. A Sahli pipet, graduated in mm.<sup>3</sup> or a Sahli pipet which has not been drawn out to a cone\* is used for the determination. The latter pipet is subdivided in 10 divisions. Thromboplastin is drawn up into the pipet to the first mark (2 mm.<sup>3</sup>) and delivered on a watch glass or into a micro test tube. With a fresh pipet 0.2 ml.<sup>3</sup> of blood (full Sahli pipet) is drawn from the finger tip and blown into the thromboplastin. After rapid mixing blood + thromboplastin are sucked back into the pipet. The mixture is now sucked back and forth within the pipet until coagulation takes place.

\* The pipets may not be dried with alcohol or acetone

With a stop watch the time interval is measured between delivery of the blood into the thromboplastin and the first sign of clotting

If the percentage of vitamin K present is to be determined, 2-3 normal blood samples must be examined in the same manner and an average value (blank value B) must be calculated

The vitamin K content in per cent is calculated by dividing the value found in the analysis (unknown U) by the average value

$$(B) \quad \frac{B}{U} \times 100 = \text{per cent vitamin K.}$$

### Example

$$\begin{array}{l} U = 24 \text{ sec.} \\ B = 19.2 \text{ sec.} \end{array} \quad \frac{19.2 \times 100}{24} = 80 \text{ per cent prothrombin.}$$

The vitamin content<sup>7</sup> is decreased in

- (1) nutritional deficiency
- (2) hypoprothrombinemia of the newborn
- (3) inadequate intestinal absorption
- (4) lack of bile in the intestine
- (5) obstructive jaundice (all types)
- (6) pyloric obstruction
- (7) injury of the liver
- (8) after treatment with 3-3 methylene-bis (4 hydroxy-coumarone) (known as dicumarone)

Latent hemorrhagic hypoprothrombinemia occurs when the prothrombin level has fallen to about 35 per cent of the normal

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## Chapter XIII

# Sulfa-Drugs, Para-Amino-Benzoic Acid and Thiocyanate

### DETERMINATION OF SULFANILAMIDE WITHOUT DEPROTEINIZATION

#### *Determination of Sulfanilamide<sup>1</sup>*

##### *(a) In serum or plasma*

*Principle of the method* A citric acid buffer, with added caffeine and urea,<sup>2</sup> is used to keep the proteins in solution. Thus the loss in the precipitate of some of the constituent being determined is avoided. The mixture has the additional advantage that the colors are developed at a constant pH thus eliminating any variations in the colors which may arise because of the indicator nature of the dye substance formed by the diazo reaction.

##### *Reagents*

- (1) N hydrochloric acid
- (2) 0.5 per cent aqueous sodium nitrite solution
- (3) buffer solution as for bilirubin-buffer solution 2a (see p 320)
- (4) 0.4 Gm. dimethyl- $\alpha$  naphthylamine is dissolved in 100 ml of 90 per cent alcohol. The solution lasts a very long time if kept on ice.
- (5) standard solution 100 mg of sulfanilamide are dissolved in a 100 ml volumetric flask in 20 ml of N/10 HCl by heating in a water bath. After cooling the solution is made up to the mark with water. If determination of a sulfanilamide derivative is wanted, a similar solution of that substance is used.

##### *Procedure*

Unknown solution 0.25 ml of serum is placed in a test tube. 0.05 ml of solution (1) and 0.05 ml of solution (2) are added. The mixture is allowed to stand for three minutes, then 2 ml of buffer-solution (3) and 0.1 ml of solution (4) is added.

Standard solution 0.70 ml of serum free from sulfanilamide, is added to 0.15 ml of solution (1), 0.15 ml of solution (2) and 0.06 ml \* of solution (5) and it is left standing for three minutes, 6 ml of the buffer solution (3) and 0.3 ml of solution (4) are added

An easily comparable, red violet, color develops immediately and is read in the Hellige Colorimeter Should the color of the unknown solution be too intense, it may be diluted with water

If the content of sulfanilamide is determined in icteric or hemolytic serum, a difference of color appears between the unknown and the standard solution This can be eliminated by putting a colorimetric cup of the diluted serum under examination behind the standard solution in a compensating colorimeter, as described in bilirubin (see p 322)

### Calculation

$$\frac{\text{mg. sulfanilamide}}{\text{per 100 ml.}} = \frac{100 - y}{100} \times 8 \quad y = \text{colorimeter reading.}$$

The factor 8 results from the following calculation 0.25 ml. of serum corresponds to 0.02 mg of the 100 mg per 100 ml sulfanilamide solution Therefore 1 ml corresponds to 0.08 mg and 100 ml. to 8 mg

If only a sulfanilamide standard is available, and another derivative is to be determined the result obtained must be multiplied by the following factor

sulfapyridin	1.47	sulfamerazine	1.54
sulfathiazole	1.48	succinyl-sulfathiazole	2.06
sulfaguanidin	1.37		
sulfadiazine	1.47		
para-amino-benzoic acid	0.8		

### (β) In urine

Besides the free sulfanilamide acetylated sulfanilamide often appears in the urine This does not take part in the reaction and can be determined only after hydrolysis with HCl

Determination of free sulfanilamide 0.25 ml of the urine, diluted 10 times 0.2 ml of N HCl and 0.05 ml of the nitrite solution

\* Using a bent pipet (0.1 ml) graduated in 1/1000th ml

are used. The further procedure and the calculation are the same as in the determination in serum. For the standard solution water is used instead of serum.

Determination of total sulfanilamide. 0.25 ml of urine diluted 10 times is boiled with 0.2 ml of N/HCl for thirty minutes in a water bath or in the steam sterilizer. After cooling 0.05 ml of sodium nitrite solution is added, and the sulfanilamide is determined as above.

The difference between total and free sulfanilamide gives the quantity of acetyl sulfanilamide in the urine.

#### (7) In cerebrospinal fluid\*

In spinal fluid sulfanilamide may also be found in the acetylated form. The determination should therefore be done as in urine, 0.2 ml of N/HCl being added to every 0.25 ml. of spinal fluid. One of the test tubes is first boiled for thirty minutes in the water bath or steam sterilizer. After cooling 0.05 ml of nitrite solution (2) is added to both tubes and the sulfanilamide content is determined. The difference between total and free sulfanilamide gives the quantity of acetyl sulfanilamide.

#### DETERMINATION OF SULFANILAMIDE AFTER DEPROTEINIZATION†

The blood filtrate—after hemolysis and deproteinizing—is diazotized and coupled with dimethyl- $\alpha$ -naphthylamine in a buffered solution.

#### Reagents

- (1) 0.5 per cent aqueous saponin solution
- (2) 15 per cent trichloroacetic acid solution
- (3) 0.1 per cent  $\text{NaNO}_2$  solution should be freshly prepared from a 10 per cent  $\text{NaNO}_2$  solution which is stable when kept on ice.

Although this color reaction is fairly specific, Long & Wood point out that the local anesthetic procaine hydrochloride (para-aminobenzoyl-diethyl amino-ethanol hydrochloride) will give a reaction similar to sulfanilamide and sulfapyridine. This is exceedingly important in the determination of levels in pleural or cerebrospinal fluids since in anesthetizing the tissues some of the solution might be introduced into the fluids and result in abnormally high readings.

† Mainly according to Bratton and Marshall, *J. Biol. Chem.* 128: 537, 1939, from J. Kolmer and F. Boerner, *Approved Laboratory Technique* 1945, p. 553.

(4) buffer solution 37.5 gm of citric acid 15 Gm of urea and 10 Gm. of caffeine are dissolved in about 75 ml of distilled water and made up to 100 ml

(5) 4 per cent alcoholic dimethyl- $\alpha$ -naphthylamine solution

(6) 100 mg per cent aqueous sulfanilamide solution

### *Procedure*

One-tenth (0.1) of a milliliter of whole blood drawn from the finger tip is placed into a short test tube, containing 1.5 ml of saponin solution (1). After standing for a short time to complete hemolysis 0.4 ml of trichloroacetic acid (2) is added and five minutes later the tube is centrifuged. One milliliter of clear supernatant is placed into a test tube to which is then added 0.1 ml of  $\text{NaNO}_2$  solution (3), and three minutes later 0.6 ml of buffer solution (4) and 0.1 ml of dimethyl- $\alpha$ -naphthylamine solution (5) are added.

**Standard solution** The following solutions are placed into a test tube in the indicated order 0.02 ml \* sulfanilamide solution (6) 3.2 ml of saponin solution (1), 0.8 ml of trichloroacetic acid (2), 0.4 ml of  $\text{NaNO}_2$  (3), 2.4 ml of buffer solution (4) and 0.4 ml of dimethyl- $\alpha$ -naphthylamine solution (5) (4 times the amount as used for the unknown sample). After standing for ten minutes the solutions are compared in the colorimeter.

### *Calculation*

$$\text{mg. per cent sulfanilamide} = \frac{\text{reading of standard} \times 10}{\text{reading of unknown}}$$

The factor 10 is derived as follows 0.05 ml of blood corresponds to 0.005 ml. of 100 mg. per cent sulfanilamide solution. Therefore 1 ml of serum corresponds to 0.1 mg and 100 ml of serum to 10 mg. (for the factor for other derivatives see p. 352). The results obtained for serum by this method are identical with the values found when working with method I. When working with whole blood, 10 per cent must be added to the result. However, this figure is not very accurate because the degree of absorption of sulfanilamide by the red cells is not constant, however, the results are informative enough.

\* Using a 0.1 ml bent pipet subdivided in 1/1000 ml (see fig. 4)

DETERMINATION OF POTASSIUM THIOCYANATE<sup>3</sup>

*Principle of the method* The protein free serum-filtrate is treated with a ferric salt solution and the produced red ferric thiocyanate is compared with a known standard solution treated in the same manner

*Reagents*

(1) 10 per cent trichloroacetic acid solution

(2) ferric nitrate solution 5 Gm of crystallized ferric nitrate are dissolved in a 100 ml. volumetric flask in about 50 ml of water, 2.5 ml of concentrated nitric acid are added and the volume made up to the mark with water

(3) standard thiocyanate solution 2.058 ml. or practically 2.00 ml of N/100 potassium thiocyanate are placed into a 100 ml. volumetric flask containing about 50 ml of distilled water 40 ml. of trichloroacetic acid (1) are added and made up to the mark with water One ml of N/100 thiocyanate solution contains 0.9718 mg KCNS therefore 2.06 ml diluted to 100 results in a solution containing 0.2 mg KCNS in 10 ml

*Procedure*

In a centrifuge tube one part of serum is diluted with two parts of distilled water To this serum dilution 2 parts of trichloroacetic acid (1) are slowly added with shaking The contents are well mixed, centrifuged and filtered Five ml of clear filtrate and 0.5 ml of ferric nitrate (2) are mixed in one tube and in a second tube 10 ml of thiocyanate standard solution (3) with 1 ml of reagent (2) After 5 minutes the developed colors are compared in a colorimeter

*Calculation*

(α) for the Duboeque colorimeter

$$\frac{\text{reading of standard}}{\text{reading of unknown}} \times 10 = \text{mg. per cent KCNS}$$

(β) for the Hellga colorimeter

$$\frac{100 - y}{100} \times 10 = \text{mg. per cent KCNS} \quad y = \text{colorimeter reading.}$$



The factor is 10 as the serum was 5 times diluted and the standard corresponds to 2 mg per cent potassium thiocyanate

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## Appendix

# Solutions and Apparatus

In order to perform a quantitative analysis the chemical constitution of the compound to be determined must be known so that a suitable method of analysis might be selected. One must also know the exact chemical reaction leading to the production of the compound to be estimated. The quantitative methods may be arranged in 4 groups

- (I) gravimetric methods
- (II) titrimetric methods
- (III) colorimetric methods
- (IV) gas-analytic methods

(I) In the gravimetric method the compound formed by a chemical reaction is estimated by weighing

For crude weighing (accuracy 0.05 Gm) a beam balance is used, for analytic weighing an analytic balance is employed when 0.1 mg may still be weighed accurately. This balance is best placed upon a stone plate resting on iron bars. The bars are let into the wall of the room. Shock absorbing media such as cork mats or sheet rubber may be placed under the plate. A good analytic balance must fulfill the following conditions. When arrested the pointer must be on the center mark of the scale (zero-point). To avoid parallax errors the distance between scale and pointer should be as short as possible. The balance must be tested for equal arm length by placing equal sized weights (50 Gm) on both sides. When the weights are exchanged, the balance must not be disturbed. If the knife edges or pans are soiled they must be cleaned with a camel hair brush or a piece of soft silk.

The balance must be protected from temperature changes, humidity and acid fumes. During the process of weighing the balance case must be kept closed and it may be reopened only after the balance has been arrested. The balance is set at zero when the deflection of the pointer to each side is equidistant from the zero point. When weighing the material to be weighed is placed on the left and the weights are placed on the right pan.

(II) Titrimetric methods are based on the estimation of the volume of a compound used up during the chemical reaction of this compound with the unknown material. Calibrated flasks and pipets are needed for the titrimetric (volumetric) estimation. Liquids are measured in

(1) measuring cylinders the accuracy of the measurement is dependent upon the length and diameter of the cylinder

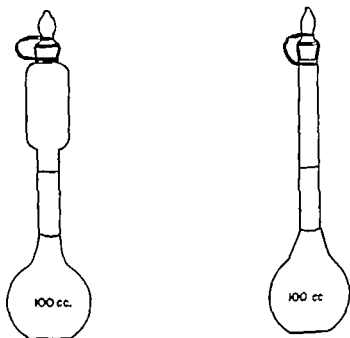


FIG. 50 VOLUMETRIC FLASKS

(2) volumetric flasks permit a more exact measurement than the cylinders. Two shapes of flasks are available

(a) flasks with neck of uniform width (fig. 50a)

(b) flasks with the necks widened above the mark (fig. 50b). This shape permits thorough mixing of the contents

(3) pipets

(a) serologic pipets. These are glass tubes with one end drawn out to a tip, sizes = 0.5 ml - 10 ml. These are subdivided as desired in 0.01th of a ml or 0.1 ml (fig. 4)

(b) bulb pipets of 4 different types are used

(α) bulb pipets with capillary tip. The capillary permits even drainage of the measured liquid. A constant amount of liquid will

always remain behind after the pipet has drained by gravity, because of capillary attraction. Therefore, this type of pipet should not be blown out.

( $\beta$ ) bulb pipet with narrowed, but not capillary tips. This type of pipet should be blown out.

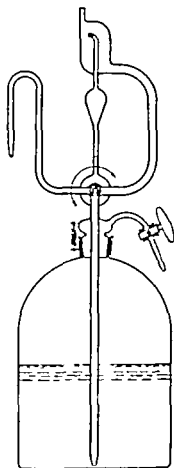


FIG. 60. AUTOMATIC PIPET

( $\gamma$ ) bulb pipets with 2 marks—the lower mark placed about 3 cm above the tip.

( $\delta$ ) bulb pipet—which permit the measurement of two different volumes (i.e. 6 ml and 15 ml). Here both marks are placed above the bulb. These pipets may be made up in the laboratory and follow the pipet is filled with water up to the original mark. Now the desired

amount of water (0.2 ml) is allowed to drain and it is weighed on an analytic balance. The new mark (5.8) is put on the pipet in the appropriate place.

(c) automatic pipets (fig 60). This type of pipet is used for consecutive measurements of the same amount of liquid volume (see measuring of potassium ferric cyanide in blood sugar determination).

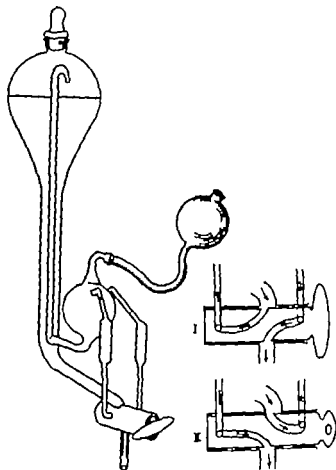


FIG 61 DEROMA PIPET

With a rubber attachment pressure is exerted pushing the liquid from a 1 liter reservoir into a capillary of known volume and with a U shaped upper end. After the first few drops have run out (to adjust the liquid in the pipet towards the top) the 3-way stopcock is turned clockwise and the contents are allowed to flow out. The use of communicating tubes assures exact automatic delivery of the fluid. The lower end of the pipet forms a capillary and is adjusted to equal height

with the lower end of the 'dropping capillary'. This arrangement permits the rapid and steady measurement of fluids.

(d) Derona pipets (fig 61) \* Depending upon the position of the stopcock one pipet is emptied while the other one fills up simultaneously.

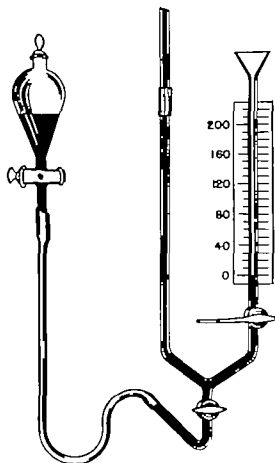


FIG 62 APPARATUS FOR THE CALIBRATION OF CAPILLARY PIPETS

(e) capillary pipets heavy walled capillary pipets with white backing are used for the measurement of small amounts of blood (0.01-0.5 ml). White-backed triangular capillary tubes may also be used one side of which is ground to serve as a lens. These pipets are easily calibrated with the apparatus shown in figure 62. The lower part of a U-shaped capillary is connected with a leveling bulb.

the U shaped tube carries a stopcock, which can interrupt the connection with the bulb. Another stopcock interrupts the connection between both arms of the U tube. The bulb is filled with a 0.1 per cent aqueous saponin solution colored with methyl violet or methylene blue. By opening both stopcocks, fluid is forced into the U tube, the stopcocks are closed and the zero point of the moveable millimeter scale, attached to the right arm, is adjusted to the meniscus of the liquid. A calibrated pipet is now attached to the left arm of the U with the aid of a short (approximately 1 cm.) piece of rubber tubing, its tip is pointed upwards, and the pipet is fastened to a ring stand with a clamp. The lower stopcock is now opened and by raising the leveling bulb the liquid is forced into the pipet until it runs out of the tip. The stopcock is closed, the outside of the pipet is wiped off and by careful opening of the right stopcock the liquid is allowed to enter from the pipet into the right arm of the U tube, until it reaches the pipet mark of the left arm. Now the stopcock is closed and the position of the upper meniscus of the liquid is marked on the millimeter scale. If both sides of the U tube are of equal width the scale can now be subdivided as desired.

The calibration of the pipet with mercury is done as follows:

Approximately 1.5 Gm. of mercury is drawn up into a capillary and the position of the mercury meniscus is marked. For the marking the pipet is coated with a thin layer of melted bees wax, and the mark is made with a file. Crude concentrated hydrofluoric acid is brushed over the mark and after thirty seconds is washed off with water. When the mark is etched in, the wax is dissolved, the mark is covered with oil paint or a colored wax pencil and the excess color is removed. The mercury is blown out into a dish and weighed on an analytical balance. The volume of the capillary is obtained by dividing the weight of the mercury by its specific gravity.

### Example

Weight of mercury = 1.6939, divided by 13.551 (specific gravity of mercury) = volume of the capillary of 0.125 ml. corresponding to a difference in height read off from the U tube of 130 mm. Therefore,

1 mm.<sup>3</sup> corresponds to  $\frac{130}{125} = 1.04$  mm. on the scale.

0.1 ml. correspond to 10.4 mm. on the scale

0.2 ml. correspond to 20.8 mm. on the scale

## (4) burettes

(a) macro-burettes are glass tubes having a volume of 25-50 ml, each ml being subdivided into tenths. Such a tube is closed at the bottom by a stopcock or a clamp. We have used Bang burettes as

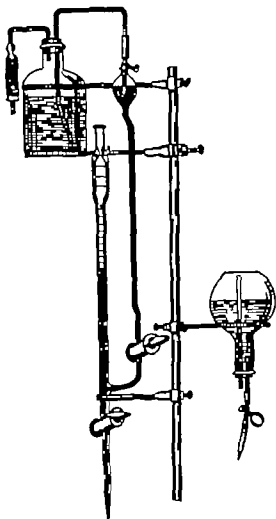


FIG. 63 Burette with reservoir and dropping bottle for starch solution

shown in figure 63 which are conveniently refilled the reservoir of the burette can be connected to a large storage flask and according to the law of communicating tubes the reservoir bulb of the burette is refilled from the storage flask.

(b) semi-micro burettes (Pregl) are constructed like macro-burettes, the burette holds 10 ml. and each milliliter is subdivided in 0.05 ml



(c) micro-burettes (Bang) The volume of the measuring tube is 3 ml, and each milliliter is subdivided in hundredths of a milliliter. The volume can be increased to 5 ml by including a wider cylindrical part on the upper end of the burette. However, the first 2 ml. are subdivided only in 0.5 ml. This type of micro-burette permits the measuring of amounts above 3 ml. with a practically unchanged length of the burette.

(d) micro-burettes according to Schwarz<sup>1</sup> (Rehberg) This burette allows the measurement of fluids between 0.5 ml and 0.01 ml with an accuracy of 0.001 ml. In contrast to the burettes described above the draining of the liquid here is not caused by gravity. The burette is filled by sucking up the liquid to the zero mark with rubber tubing attached to a calcium chloride tube (k). The liquid can not drain spontaneously, because the liquid level in the measuring part of the burette is lower than in the tip of the burette. Air can not enter the burette, because the height of the capillary, corresponding to the inner diameter of the tip, is larger than the difference in height between a and b (end of tip and graduation). In this manner the solution is kept stationary. In order to titrate the tip of the burette is dipped into the liquid to be titrated by pressing the moveable key of the burette stand. The titration liquid is forced out by blowing air through k. When the end point is reached, blowing is interrupted, the key is released, lifting the tip of the burette out of the liquid and the flow of liquid is interrupted. If the tip would remain immersed in the flask, the solution would be sucked back into the burette. After the titration a small test tube is slipped over the tip of the burette, holding it in place with a rubber stopper. This protects the opening from dust (fig. 64 a and b).

(e) Instead of working with the Schwarz-Rehberg burette, a 0.2 ml pipet may be used which is subdivided in 1/1000 of a milliliter. The tip of the pipet is bent and beveled as shown in figure 65. The liquid employed for titration is sucked up while holding the pipet with the right hand and the flask with the left hand (see fig. 65). The liquid is allowed to drain along the wall of the flask and the flask is carefully shaken, so that the tip of the pipet does not touch the contents of the flask.

If a large number of titrations has to be performed this same pipet can easily be converted into a burette (fig. 66) by mounting it on a

small iron stand and connecting the upper end with a piece of glass tubing, using rubber tubing as connection. A wide rubber tube is attached to the end of the glass tubing, it is filled with mercury and closed off with a rubber stopper. The rubber tubing carries a screw clamp. To fill the burette the rubber tubing is compressed by closing the screw clamp thus ejecting all air present in the system. The

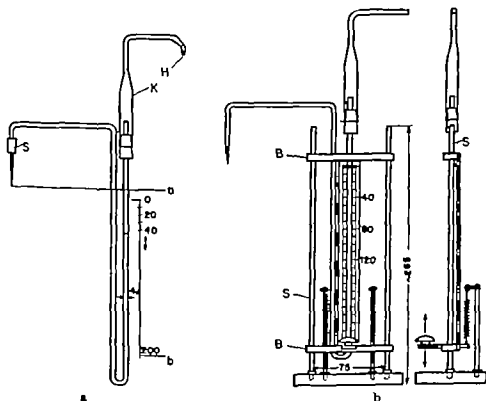


FIG. 64 Micro-burette according to Schwarz (Rehberg)

burette is now dipped into the solution, used for titration and by careful manipulation of the clamp the burette may be filled as described. For the titration the tip of the burette should touch the inner wall of the vessel containing the sample and the screw is turned allowing the solution to flow out until the color change is obtained.

All volumetric flasks, pipets and burettes should be of standard glassware or calibrated by weighing with water and calculating the corrected volume with the aid of table 1.

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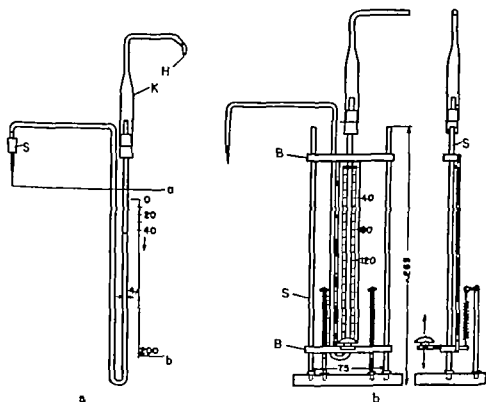


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burette is now dipped into the solution, used for titration and by careful manipulation of the clamp the burette may be filled as described. For the titration the tip of the burette should touch the inner wall of the vessel, containing the sample and the screw is turned allowing the solution to flow out until the color change is obtained.

All volumetric flasks, pipets and burettes should be of standard glassware or calibrated by weighing with water and calculating the corrected volume with the aid of table 1.

*Examples*

(1) What weight of water has to be taken at 30° to calibrate a liter flask?

*Answer* Reading in the table 1, opposite 30° the value 994.92 Gm. is found This is the weight of water required

(2) What amount of water has to be weighed at 20° to calibrate a 250 ml flask?

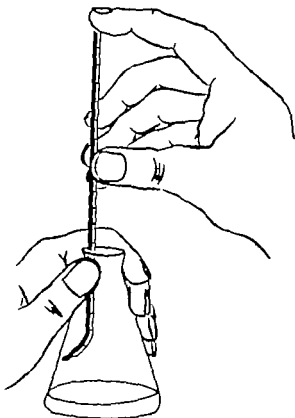


FIG. 65 Position of hands for pipetting

*Answer* Value for 1000 ml at 20° = 997.18 Gm Therefore for the volume 250 ml,  $\frac{250 \times 997.18}{1000} = 249.29(5)$  Gm of water is required

All glassware must be free from fat its presence is recognized by the way liquid adheres to the glass walls For cleaning soap and water, or alcoholic alkali is used It is best, however to use chromic acid-sulfuric acid a solution of 200 Gm of technical sodium or potassium dichromate in 1000 ml of concentrated sulfuric acid The acid

is left overnight in the vessels to be cleaned. Pipets only need an occasional acid cleaning. Ordinarily they are washed with water and rinsed with acetone. The acetone removes all traces of fat and at the same time serves to dry the pipets. To speed up drying the pipets

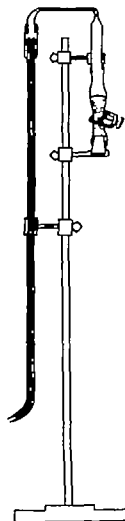


FIG. 66 Pipet converted into burette

while connected to a water suction pump may be drawn through the cold part of a bunsen burner or the apparatus as shown in figure 67 is used. This consists of a nickel-coated copper block, which is continuously heated by two micro-burners. The copper block heats up

only moderately, which prevents cracking of the pipets and also keeps their tips from being damaged, an accident that may happen to beginners

If gas is not available, the block can be heated by building small electrical resistances into the apparatus. It is recommended to incorporate a colored signal light as a reminder to shut off the current, when the block is not in use

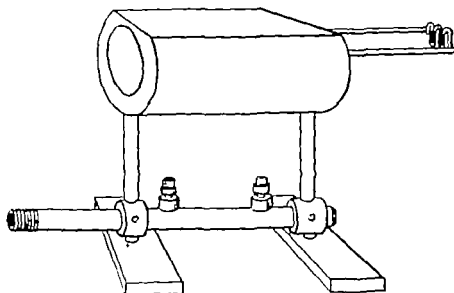


FIG. 87 APPARATUS FOR DRYING PIPETS

This oven also serves for quick drying of test tubes. The test tubes are placed inside the oven and by means of a pipet connected to a water suction pump the evaporating water inside the test tubes is quickly removed

Quick drying of small test tubes or centrifuge tubes can also be achieved by centrifuging the cleaned tubes on absorbent cellulose in an upside down position

#### PREPARATION OF STANDARD SOLUTIONS

A normal solution contains 1 Gm equivalent (referring to hydrogen as 1) of material dissolved in 1 liter of solvent

N/1 hydrochloric acid contains 36.5 Gm of HCl dissolved in 1 liter of water  $\left(\frac{\text{mol. weight}}{1}\right)$  N/1 sulfuric acid contains 49.045 Gm.

$\text{H}_2\text{SO}_4$  dissolved in 1 liter of water  $\left(\frac{\text{mol weight}}{2}\right)$  N/1 potassium iodate contains 3.671 Gm of  $\text{KIO}_3$  dissolved in 1 liter of water  $\left(\frac{\text{mol weight}}{6}\right)$  as 3 atoms of oxygen = 6 atoms of hydrogen are liberated upon decomposition

This shows that one unit of volume of a N/1 acid, regardless of the valence of the acid, corresponds to one unit of volume of alkali, regardless of its valence

*Preparation of N/10 Potassium Iodate Solution (Primary Standard)*

In a 1 liter volumetric flask 3.567 Gm of potassium iodate (anhydrous, purest grade) dried to constant weight in a desiccator or in an oven are dissolved in water and made up to the mark with water. This substance is well suited as primary standard for the standardization of all other substances used

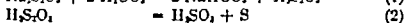
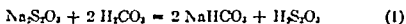


With equal amounts of iodate present, the amount of liberated iodine depends upon the acidity of the solution. Therefore acids as well as sodium thiosulfate solutions can be standardized directly with iodate solutions. The titer of alkali solutions, potassium permanganate solutions etc. can then be indirectly determined

The N/10 solution can be diluted to give N/20, N/40, N/80, N/100 solutions. The dilute  $\text{KIO}_3$  solutions, used for the determination of acid or alkali must be freshly prepared before use. When the potassium iodate solution is acidified with dilute  $\text{H}_2\text{SO}_4$  as for the titration of permanganate and thiosulfate, it will keep for a long period of time

*Preparation of N/10 Sodium Thiosulfate Solution*

Approximately 20 Gm (theoretical amount needed 21.800 Gm) of sodium thiosulfate (purest grade) is dissolved in freshly boiled distilled water in a 1 liter volumetric flask and made up to the mark with water. A small amount of thiosulfate is decomposed by traces of  $\text{CO}_2$  present in water forming sulfurous acid





only moderately, which prevents cracking of the pipets and also keeps their tips from being damaged, an accident that may happen to beginners

If gas is not available, the block can be heated by building small electrical resistances into the apparatus. It is recommended to incorporate a colored signal light as a reminder to shut off the current, when the block is not in use

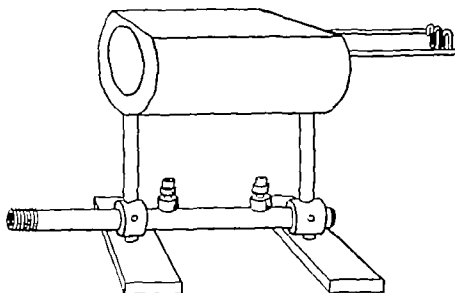


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#### *Preparation of N/10 Potassium Iodate Solution (Primary Standard)*

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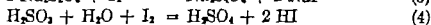
With equal amounts of iodate present, the amount of liberated iodine depends upon the acidity of the solution. Therefore acids as well as sodium thiosulfate solutions can be standardized directly with iodate solutions. The titer of alkali solutions, potassium permanganate solutions etc. can then be indirectly determined.

The N/10 solution can be diluted to give N/20 N/40 N/50 N/100 solutions. The dilute  $\text{KIO}_3$  solutions, used for the determination of acid or alkali must be freshly prepared before use. When the potassium iodate solution is acidified with dilute  $\text{H}_2\text{SO}_4$  as for the titration of permanganate and thiosulfate, it will keep for a long period of time.

#### *Preparation of N/10 Sodium Thiosulfate Solution*

Approximately 20 Gm. (theoretical amount needed 24.805 Gm.) of sodium thiosulfate (purest grade) is dissolved in freshly boiled distilled water in a 1 liter volumetric flask and made up to the mark with water. A small amount of thiosulfate is decomposed by traces of  $\text{CO}_2$  present in water forming sulfurous acid





According to equation (4) increasing amounts of iodine are used up by the thiosulfate solution during the first few days (equation 2). When all  $\text{CO}_2$  present in water has reacted (usually after several days), the solution will be stable for a long period of time, when kept in a cool and dark place. It is best to prepare a large volume of thiosulfate solution and determine its titer approximately 10-14 days later as follows. 25 ml of N/10  $\text{KIO}_3$  solution is acidified with dilute  $\text{H}_2\text{SO}_4$ , approximately 10 ml of a 10 per cent KI solution are added, and the liberated iodine is titrated with thiosulfate from a macroburette using starch as indicator.

Example of a calculation. 25 ml of N/10  $\text{KIO}_3$  solution use up 24.8 ml of thiosulfate solution. Accordingly, the thiosulfate is the stronger solution and all values determined with this thiosulfate must be multiplied by the factor  $\frac{25}{24.8}$  to give the correct result. When,

N/100 or N/200 thiosulfate is to be prepared from this stock solution, 9.92 ml of N/10 thiosulfate is diluted to 100 ml or 200 ml with water (to avoid using the factor  $\frac{25}{24.8} = \frac{10}{X}$ ), or 9.92 ml of stock solution is diluted to 1 liter. This is done as follows. A 1000 ml volumetric flask is filled to the mark with thiosulfate stock solution the excess fluid (8 ml in this case) is removed with a pipet and the volume is made up again to 1000 ml with water. All dilute thiosulfate solutions will keep only for a limited time. The N/100 or N/200 thiosulfate solutions may be stabilized (several weeks) by the addition of 6 ml of 4 per cent (N/1) NaOH for each liter. This alkaline thiosulfate solution may only be employed when the titration is performed in strongly acid medium. It may not be used for titration in neutral medium because of the formation of hypoiodide. This would be the case in the titrimetric (acidimetric) determination of nitrogen. The acid liquid in the receiver flask is neutralized by the added  $\text{KIO}_3$  and KI and during titration with alkaline thiosulfate the side reaction, mentioned above, would take place.

#### Preparation of Starch Solution

Two and one-half (2.5) grams of soluble starch is suspended in some cold water in a 1 liter Florence flask, one liter of boiling water is added

and it is heated to boiling 3 times. This starch solution must be stabilized with  $\text{HgI}_2$  either in the form of rods or celluloid plates.<sup>3</sup>

(a) preparation of  $\text{HgI}_2$ -rods A paste is prepared from  $\text{HgI}_2$  and Mastisol, and wooden rods (applicators) are covered with the paste. When dry the rods are soaked in water for twenty four hours and again dried.

(b) preparation of  $\text{HgI}_2$  plates Celluloid strips or cleaned used (inflammable) X ray film strips are dissolved in acetone, mixed with  $\text{HgI}_2$ -powder and the solution is poured into petri-dishes. Small plates will form, which are dried soaked in water for twenty four hours and again dried. One of these rods or plates is placed in the starch solution for stabilization.  $\text{HgI}_2$  does not interfere with the iodometric titration. The rods or plates will maintain their activity for a long time and may be used over again for fresh starch solution. They must be discarded when the color begins to fade out.

For use during the titration the starch solution is placed in flasks which are slipped through a ring the neck pointing down. The flask is closed with a 2 hole rubber stopper equipped with 2 glass tubes. One tube reaches to the bottom of the flask the second tube is short and is connected to a capillary with rubber tubing carrying a clamp. Thus the starch is protected from dust and it is easier to handle than a dropping bottle (fig. 63).

#### *N/10 Iodine Solution*

In a 1 liter flask approximately 25 Gm of KI (iodate-free) is dissolved in some water and 12.7 Gm of iodine (weighed on a watch glass on a beam balance) is added. It is well shaken until the salt has dissolved and made up to the mark with water. In order to determine the titer 25 ml of this solution is pipeted into a flask, acidified with a small amount of dilute  $\text{HCl}$  or  $\text{H}_2\text{SO}_4$  and titrated with N/10 sodium thiosulfate solution. For the calculation of the factor see thio-sulfate titration.

This solution is not stable. Using an acidified N/10 potassium iodate solution it is possible to prepare the N/10 potassium iodine solution by adding a few crystals of KI directly before use (see also under micro-acetone, p. 275).

#### *N/1 Hydrochloric Acid*

In a 1 liter volumetric flask approximately 100 ml of fuming  $\text{HCl}$  (specific gravity 1.10) is made up to the mark with water. To deter-

mine the factor a few crystals of  $\text{KIO}_3$  and  $\text{KI}$  are added to 5 ml of the acid and the liberated iodine is titrated with  $\text{N}/10$  neutral thio-sulfate till yellow, then after the addition of starch the titration is continued until colorless

### Example

5 ml of  $\text{HCl}$  use up 52 ml of  $\text{N}/10$  thiosulfate solution Therefore, the acid is stronger than  $\text{N}/1$  The equation  $\frac{50}{52} = \frac{X}{1000}$ ,  $X = \frac{50 \times 1000}{52} = 961.5$  shows, that 961.5 ml of acid must be diluted to 1000 ml with water in order to obtain an exactly  $\text{N}/1$   $\text{HCl}$  (see under *N/10 Sodium Thiosulfate Solution* for measuring of 961.5 ml)

### Preparation of N/1 Sulfuric Acid

Into a 1 liter volumetric flask containing distilled water 28 ml of concentrated  $\text{H}_2\text{SO}_4$  (specific gravity 1.84) is poured, and after cooling made up to the mark with water For the determination of the factor see under *N/1 Hydrochloric Acid* If  $2/3$   $\text{N}$   $\text{H}_2\text{SO}_4$  is desired, 200 ml of  $\text{N}/1$   $\text{H}_2\text{SO}_4$  is diluted to 300 ml in a volumetric flask

### Preparation of N/1 Sodium Hydroxide

In a porcelain dish approximately 42 Gm of  $\text{NaOH}$  (purest grade) is dissolved in distilled water and after cooling is transferred to a 1 liter volumetric flask, where the volume is made up to the mark with water After the addition of a few drops of methyl red as indicator, 20 ml of alkali are titrated with  $\text{N}/1$   $\text{HCl}$  or  $\text{H}_2\text{SO}_4$  until the color changes to red

### Example

20 ml of alkali require 20.8 ml of acid for neutralization. The alkali is stronger than the acid and must be diluted according to the formula

$$\frac{20}{20.8} = \frac{X}{1000}, \quad X = \frac{20 \times 1000}{20.8} = 961.5$$

Therefore 961.5 ml of alkali must be made up to 1000 ml with water

*Preparation of Methyl Red Indicator*

In 100 ml. of hot water 0.02 Gm. of p-dimethylamino-azo-benzene-o-carboxylic acid  $(\text{CH}_3)_2\text{N}-\text{C}_6\text{H}_4-\text{N}=\text{N}-\text{C}_6\text{H}_4-\text{COOH}$  is dissolved and after cooling filtered into a dropping bottle, 2-3 drops of this solution are used for titration.

The following indicator mixture may be used instead of methyl red. 80 ml. of saturated alcoholic methyl red solution are mixed with 20 ml. of 1 per cent alcoholic methylene-blue solution. The color change is well defined, from green (alkaline) to purple (acid).

*Preparation of N/1 Potassium Hydroxide*

In a porcelain dish approximately 60 Gm. of KOH is dissolved in some water. After cooling this solution is transferred to a 1 liter volumetric flask and made up to the mark with distilled water. The factor of the solution is determined as described for sodium hydroxide (page 372).

*Preparation of N/1 Sodium Carbonate Solution*

On an analytical balance exactly 53.0 Gm. of anhydrous  $\text{Na}_2\text{CO}_3$  (purest grade) are weighed and made up with distilled water to 1 liter in a volumetric flask. This solution requires no titer.

*Preparation of Molar Phosphoric Acid*

One molecule of  $\text{H}_3\text{PO}_4$  reacts with one molecule of NaOH to form one molecule of primary sodium phosphate. Using methyl-orange (saturated aqueous solution) or bromocresol green (0.1 per cent in 20 per cent alcohol) as indicator the first drop in excess of NaOH causes a color change to yellow or to blue-green respectively, by forming secondary phosphate.



In a volumetric flask approximately 60 Gm. of syrupy phosphoric acid (specific gravity 1.70) is diluted with distilled water to 1 liter. Twenty milliliters of this solution is measured out into a small Erlenmeyer flask and after the addition of several drops of aqueous methyl-orange solution it is titrated with N/1 NaOH until a yellow color appears. The addition of 5-6 Gm. of pure NaCl will help to give a sharp end point.

*Example*

$$S_1 = 15 \text{ mm}$$

$$C_1 = 4 \text{ mg per cent}$$

$$S_2 = 18 \text{ mm}$$

$$\frac{S_1}{S_2} \text{ (from table 24) } = 0.833$$

$$C_2 = 0.833 \times 4 = 3.332 \text{ mg per cent}$$

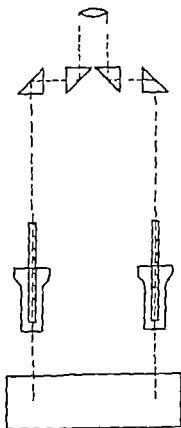


FIG. 68 Principle of the Dubosque colorimeter

(2) *Hellige colorimeter (fig 69)* This colorimeter provides a standard cup for the unknown solution. The depth of the layer is varied by moving a wedge filled with the standard solution until equal color intensity is achieved. The colors are observed through a small slit. The position of the wedge is read off on a scale, which is subdivided into 100 divisions. The zero point of the scale corresponds to the thickest part of the wedge and the setting at the 100 point is practically colorless. The wedge, which is filled with standard solution must be calibrated. The best light source is diffuse daylight. Tables p. 10

TABLE 21.—Factors  $S_1/S_0$  for the Dubosque collimator  $S_1 = 15$  and 20

$S_0$	$S_1$	00	01	02	03	04	05	06	07	08	09
15	0.2	2.00	2.40	2.81	3.22	3.62	4.02	4.42	4.82	5.22	5.62
15	0.3	3.33	3.99	4.65	5.31	5.97	6.63	7.29	7.95	8.61	9.27
15	0.4	4.66	5.61	6.56	7.51	8.46	9.41	10.36	11.31	12.26	13.21
15	0.5	5.99	7.21	8.43	9.65	10.87	12.09	13.31	14.53	15.75	16.97
15	0.6	7.32	8.81	10.29	11.78	13.26	14.75	16.23	17.72	19.20	20.68
15	0.7	8.65	10.41	12.17	13.93	15.69	17.45	19.21	20.97	22.73	24.49
15	0.8	9.98	12.01	14.04	16.07	18.10	20.13	22.16	24.19	26.22	28.25
15	0.9	11.31	13.60	15.89	18.18	20.47	22.76	25.05	27.34	29.63	31.92
15	1.0	12.64	15.21	17.82	20.43	23.04	25.65	28.26	30.87	33.48	36.09
15	1.1	13.97	16.84	19.55	22.36	25.17	27.99	30.81	33.69	36.57	39.25
15	1.2	15.30	18.47	21.18	24.09	27.00	29.91	32.92	36.03	39.14	42.25
15	1.3	16.63	20.10	22.81	25.82	28.61	31.72	34.93	38.24	41.55	45.15
15	1.4	17.96	21.73	24.44	27.65	30.42	33.53	37.04	40.65	44.16	48.06
15	1.5	19.29	23.36	26.07	29.48	32.23	35.34	39.15	43.16	46.77	50.97
15	1.6	20.62	24.99	27.70	31.31	34.03	37.15	41.26	45.27	49.28	53.88
15	1.7	21.95	26.62	29.33	33.14	35.82	39.06	43.37	47.38	51.39	56.79
15	1.8	23.28	28.25	30.96	34.97	37.61	40.91	45.48	49.49	53.50	59.70
15	1.9	24.61	29.88	32.59	36.80	39.30	42.82	47.59	51.60	55.61	62.61
15	2.0	25.94	31.51	34.22	38.63	41.00	44.73	49.70	53.71	57.72	65.52
15	2.1	27.27	33.14	35.85	40.46	42.70	46.84	51.81	55.82	59.83	68.43
15	2.2	28.60	34.77	37.48	42.29	44.59	48.95	53.92	57.93	61.94	71.34
15	2.3	29.93	36.40	39.11	44.12	46.42	51.06	56.03	60.04	64.05	74.25
15	2.4	31.26	38.03	40.74	45.95	48.25	53.17	58.14	62.15	66.16	77.16
15	2.5	32.59	39.66	42.37	47.78	50.04	55.28	60.25	64.26	68.27	80.07
15	2.6	33.92	41.29	44.00	49.61	51.93	57.39	62.36	66.37	70.38	82.98
15	2.7	35.25	42.92	45.63	51.44	53.82	59.50	64.47	68.48	72.49	85.89
15	2.8	36.58	44.55	47.26	53.27	55.65	61.61	66.58	70.59	74.60	88.80
15	2.9	37.91	46.18	48.89	55.10	57.50	63.72	68.73	72.74	76.75	91.71
15	3.0	39.24	47.81	50.52	56.93	59.33	65.85	70.86	74.87	78.88	94.62
15	3.1	40.57	49.44	52.15	58.76	61.14	67.98	73.09	77.10	81.11	97.53
15	3.2	41.90	51.07	53.78	60.59	62.93	69.89	75.20	79.21	83.22	100.44
15	3.3	43.23	52.70	55.41	62.42	64.82	71.80	76.81	80.82	84.83	103.35
15	3.4	44.56	54.33	57.04	64.31	66.71	73.71	78.72	82.73	86.74	106.26
15	3.5	45.89	55.96	58.67	66.20	68.60	75.62	80.63	84.64	88.65	109.17
15	3.6	47.22	57.59	60.30	68.09	70.49	77.53	82.54	86.55	90.56	112.08
15	3.7	48.55	59.22	61.93	69.98	72.38	79.46	84.47	88.48	92.49	114.99
15	3.8	49.88	60.85	63.56	71.87	74.27	81.35	86.36	90.37	94.38	117.90
15	3.9	51.21	62.48	65.19	73.76	76.06	83.24	88.25	92.26	96.27	120.81
15	4.0	52.54	64.11	66.82	75.65	77.95	85.13	90.14	94.15	98.16	123.72
15	4.1	53.87	65.74	68.45	77.54	79.84	87.02	92.03	96.04	100.05	126.63
15	4.2	55.20	67.37	70.08	79.43	81.73	88.91	93.92	97.93	101.94	129.54
15	4.3	56.53	69.00	71.71	81.32	83.62	90.80	95.81	99.82	103.83	132.45
15	4.4	57.86	70.63	73.34	83.21	85.51	92.69	97.70	101.71	105.72	135.36
15	4.5	59.19	72.26	74.97	85.10	87.40	94.58	99.59	103.60	107.61	138.27
15	4.6	60.52	73.89	76.60	86.99	89.29	96.47	101.48	105.49	109.50	141.18
15	4.7	61.85	75.52	78.23	88.88	91.18	98.36	103.37	107.38	111.39	144.09
15	4.8	63.18	77.15	79.86	90.77	92.87	100.25	105.26	109.27	113.30	147.00
15	4.9	64.51	78.78	81.49	92.66	94.56	102.14	107.15	111.16	115.21	149.91
15	5.0	65.84	80.41	83.12	94.55	96.45	104.03	109.04	113.05	117.12	152.82
15	5.1	67.17	82.04	84.75	96.44	98.34	105.92	110.93	114.94	119.03	155.73
15	5.2	68.50	83.67	86.38	98.33	100.23	107.81	112.83	116.83	120.94	158.64
15	5.3	69.83	85.30	88.01	100.22	102.12	109.70	114.72	118.72	122.85	161.55
15	5.4	71.16	86.93	89.64	102.11	104.01	111.59	116.61	120.61	124.76	164.46
15	5.5	72.49	88.56	91.27	104.00	105.90	113.48	118.50	122.50	126.67	167.37
15	5.6	73.82	90.19	92.90	105.89	107.79	115.37	120.39	124.39	128.58	170.28
15	5.7	75.15	91.82	94.53	107.78	109.68	117.26	122.28	126.28	130.49	173.19
15	5.8	76.48	93.45	96.16	109.67	111.57	119.15	124.17	128.17	132.40	176.10
15	5.9	77.81	95.08	97.79	111.56	113.46	121.04	126.06	130.06	134.31	179.01
15	6.0	79.14	96.71	99.42	113.45	115.35	122.93	127.95	131.95	136.22	181.92
15	6.1	80.47	98.34	101.05	115.34	117.24	124.82	129.84	133.84	138.13	184.83
15	6.2	81.80	99.97	102.68	117.23	119.13	126.71	131.73	135.73	140.04	187.74
15	6.3	83.13	101.60	104.31	119.12	121.02	128.60	133.62	137.62	141.95	190.65
15	6.4	84.46	103.23	105.94	121.01	122.91	130.49	135.51	139.51	143.86	193.56
15	6.5	85.79	104.86	107.57	122.90	124.80	132.38	137.40	141.40	145.77	196.47
15	6.6	87.12	106.49	109.20	124.79	126.69	134.27	139.29	143.29	147.68	199.38
15	6.7	88.45	108.12	110.83	126.68	128.58	136.16	141.18	145.18	149.59	202.29
15	6.8	89.78	109.75	112.46	128.57	130.47	138.05	143.07	147.07	151.50	205.20
15	6.9	91.11	111.38	114.09	130.46	132.36	140.04	144.96	148.96	153.41	208.11
15	7.0	92.44	113.01	115.72	132.35	134.25	141.93	146.85	150.85	155.32	211.02
15	7.1	93.77	114.64	117.35	134.24	136.14	143.82	148.74	152.74	157.23	213.93
15	7.2	95.10	116.27	118.98	136.13	138.03	145.71	150.63	154.63	159.14	216.84
15	7.3	96.43	117.90	120.61	138.02	140.02	147.60	152.52	156.52	161.05	219.75
15	7.4	97.76	119.53	122.24	139.91	141.91	149.49	154.41	158.41	162.96	222.66
15	7.5	99.09	121.16	123.87	141.80	143.80	151.38	156.30	160.30	164.87	225.57
15	7.6	100.42	122.79	125.50	143.69	145.69	153.27	158.19	162.19	166.78	228.48
15	7.7	101.75	124.42	127.13	145.58	147.58	155.16	160.08	164.08	168.69	231.39
15	7.8	103.08	126.05	128.76	147.47	149.47	157.05	161.97	165.97	170.60	234.30
15	7.9	104.41	127.68	130.39	149.36	151.36	158.94	163.86	167.86	172.51	237.21
15	8.0	105.74	129.31	132.02	151.25	153.25	160.83	165.75	169.75	174.42	240.12
15	8.1	107.07	130.94	133.65	153.14	155.14	162.72	167.64	171.64	176.33	243.03
15	8.2	108.40	132.57	135.28	155.03	157.03	164.61	169.53	173.53	178.24	245.94
15	8.3	109.73	134.20	136.91	156.92	158.92	166.50	171.42	175.42	180.15	248.85
15	8.4	111.06	135.83	138.54	158.81	160.81	168.39	173.31	177.31	182.06	251.76
15	8.5	112.39	137.46	140.17	160.70	162.70	170.28	175.20	179.20	183.97	254.67
15	8.6	113.72	139.09	141.80	162.59	164.59	172.17	177.09	181.09	185.88	257.58
15	8.7	115.05	140.72	143.43	164.48	166.48	174.06	178.98	182.98	187.79	260.49
15	8.8	116.38	142.35	145.06	166.37	168.37	175.95	180.87	184.87	189.70	263.40
15	8.9	117.71	143.98	146.69	168.26	170.26	177.84	182.76	186.76	191.61	266.31
15	9.0	119.04	145.61	148.32	170.15	172.15	179.73	184.65	188.65	193.52	269.22
15	9.1	120.37	147.24	149.95	172.04	174.04	181.62	186.54	190.54	195.43	272.13
15	9.2	121.70	148.87	151.58	173.93	175.93	183.51	188.43	192.43	197.34	275.04
15	9.3	123.03	150.50	153.21	175.82	177.82	185.40	190.32	194.32	199.25	277.95
15	9.4	124.36	152.13	154.84	177.71	179.71	187.29	192.21	196.21	201.16	280.86
15	9.5	125.69	153.76	156.47	179.60	181.60	189.18	194.10	198.10	203.07	283.77
15	9.6	127.02	155.39	158.10	181.49	183.49	191.07	195.99	200.00	204.98	286.68
15	9.7	128.35	157.02	159.73	183.38	185.38	192.96	197.88	201.89	206.89	289.59
15	9.8	129.68	158.65	161.36	185.27	187.27	194.85	199.77	203.78	208.80	292.50
15	9.9	131.01	160.28	162.99	187.16	189.16	196.74	201.66	205.67	210	



vided by the Firm Helligo and furnished together with each wedge are used for the calculations, or the tables have to be compiled in the laboratory. The latter is done by filling the cup with solutions of various, known concentrations, comparing them with the wedge and noting the colorimeter scale readings. The concentrations are marked on the ordinate and the readings on the abscissae of a graph system and the standard curve for this particular wedge is obtained by connecting all the points. From this curve any concentration

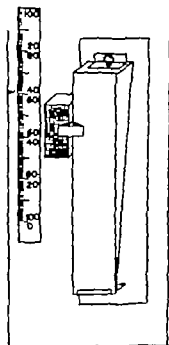


FIG. 69 PRINCIPLE OF THE HELLIGO COLORIMETER

corresponding to the color intensity can be read off for each scale division.

If the volume of the solution to be tested is too small, the cup can be made smaller as described for bilirubin, chapter X (fig. 53).

When working with color reactions which obey Beer's law, a standard solution may be used in the same way as described for the Dubouque colorimeter.

*Calculation*

$$C_x = \frac{(100 - y) \times C_1}{100}$$



and compared with the material to be analyzed. The concentration of the unknown sample can be read off directly from the standard tube which compares most nearly in color with the unknown. If the colors do not match exactly, the concentration can be determined by interpolation.

The comparator consists of a wooden block constructed as follows. In the front of the block are 3 cylindrical holes which reach to the back portion of the block. This back wall is made of opaque glass. On the top of the block are 6 cylindrical holes, arranged in pairs, each horizontal cylinder intersecting with 2 vertical cylinders. It is most important that the thickness of the walls and the diameter of all test tubes used be identical. This colorimeter permits comparison of colored samples, where the prepared colored standard does not take part in the reaction. When each of the color components (constant standard solution and colored unknown sample) is placed in separate tubes in paired holes of the block, the resultant color mixture can be compared with a single solution containing both components. This colorimeter is mainly used for the determination of hydrogen ion concentrations.

#### DETERMINATION OF HYDROGEN ION CONCENTRATION (ACCORDING TO MICHAELIS)

Aqueous solutions of acids, alkalis, and salts contain these materials partly as molecules, partly in dissociated form. The dissociated (ionized) components are either positively (cations) or negatively (anions) charged. Compounds which are dissociated in aqueous solutions are called electrolytes. The acidity of a solution is measured by the amount of dissociated hydrogen ions present in 1 liter and expressed in grams. One liter of water contains  $10^{-7}$  Gm (0.0000001 Gm) of dissociated H ions. Solutions which contain an equal amount of dissociated H ions per liter are called neutral solutions. Solutions containing less than  $10^{-7}$  Gm of H ions per liter are alkaline, solutions containing more than  $10^{-7}$  Gm of H ions per liter are acid. If a solution is N/10000 with regard to its hydrogen ions present, the concentration is expressed as follows:

$$C_H \text{ (symbol for H ion concentr)} = \frac{1}{10000} = \frac{1}{10^4} = 10^{-4} \text{ Gm.}$$



*Indicators used*

$\alpha$ -dinitrophenol for pH	between 2.8-4.8
$\gamma$ -dinitrophenol for pH	between 4.0-5.4
p-nitrophenol for pH	between 5.2-7.0
m nitrophenol for pH	between 6.0-8.4

A stock solution of each indicator is prepared and used according to the acidity of the solution to be tested

*Reagents*

- (1) 0.05 per cent aqueous solution of  $\alpha$ -dinitrophenol (1,2,4)
- (2) 0.025 per cent aqueous solution of  $\gamma$ -dinitrophenol
- (3) 0.1 per cent aqueous solution of p-nitrophenol
- (4) 0.3 per cent aqueous solution of m nitrophenol
- (5) N/10 sodium carbonate solution
- (6) 0.85 per cent NaCl solution

According to the modification of Reino Haemaelainen, E. E. Leikola and Y. Airila<sup>4</sup> one series of dye solutions can be prepared to compare colors produced by various indicators, which give identical colors, but in different pH ranges. This series of dye solutions is prepared from N/10 sodium carbonate and a dilute  $\alpha$ -dinitrophenol solution according to the plan shown below

In a 100 ml volumetric flask 10 ml of aqueous dinitrophenol solution are placed and made up to the mark with N/10 sodium carbonate solution

*Dilution series*

$\alpha$ -dinitrophen. dilute sol ml	0	51	0	78	1	20	1	74	2	50	3	40	4	60	5	70	6	70				
$\alpha$ -dinitrophen con centr sol ml																		0	77	0	85	
N/10 sod carb sol ml	6	49	6	22	5	80	5	26	4	50	3	60	2	40	1	30	0	30	6	23	6	15
tube no	I	II	III	IV	V	VI	VII	VIII	IX	X	XI											

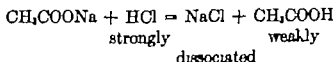
When equal color intensity is obtained in the standard and sample tube, the pH value listed below is reached according to the indicator used for the determination (table 25)



To 10 ml of sample is added 0.1 ml of indicator mixture. The colors observed correspond to the following pH

color of the solution	pH
pink	2.0
orange red	3.0
orange	4.0
yellow-orange	5.0
lemon yellow	6.0
greenish yellow	7.0
green	8.0
greenish blue	9.0
purple	10.0
reddish purple	11.0

When a mineral acid is added slowly to a solution of the salt of a strong acid and a strong base, e.g., sodium sulfate, the pH changes rapidly, according to the actual acidity of the solution. If, however, the same acid is added to an equal concentration of a solution of the salt of a weak acid and a strong base, e.g., sodium acetate, the pH rises gradually, according to the pH of the weak acid, liberated by the reaction of the strong acid with the salt



The effect of salts of weak acids upon the influence of strong acids, and vice versa, the effect of salts of weak bases with strong acids upon the influence of strong alkali is called buffer effect.

Buffer mixtures as described by Soerensen are used for the preparation of solutions of known pH.

*Standard solutions according to Soerensen*

*Solutions used (see tables following)*

- (1) N/10 HCl
- (2) N/10 NaOH
- (3) N/10 Glycine + N/10 NaCl (7.5 Gm of glycine and 5.85 Gm of NaCl dissolved in 1 liter of water)
- (4) 1/15 molar primary potassium phosphate 9.078 Gm of  $\text{KH}_2\text{PO}_4$  in 1 liter of water
- (5) 1/15 molar secondary sodium phosphate 11.876 Gm of  $\text{Na}_2\text{HPO}_4 \times 2 \text{H}_2\text{O}$  are dissolved in 1 liter of water

TABLE 26 Acid Glycine Mixtures

		pH
10.00 ml glycine		6.10 <sub>6</sub>
9.00	+ 0.10 ccm HCl	4.41
8.75	+ 0.25	3.09 <sub>1</sub>
8.50	+ 0.50	3.67 <sub>2</sub>
8.00	+ 1.00	3.34 <sub>1</sub>
8.00	+ 2.00	2.92 <sub>1</sub>
7.00	+ 3.00	2.60 <sub>7</sub>
6.00	+ 4.00	2.27 <sub>1</sub>
5.00	+ 5.00	1.93 <sub>1</sub>
4.00	+ 6.00	1.64 <sub>1</sub>
3.00	+ 7.00	1.41 <sub>1</sub>
2.00	+ 8.00	1.25 <sub>1</sub>
1.00	+ 9.00	1.14
	+ 10.00	1.03 <sub>1</sub>

TABLE 27 Alkaline Glycine Mixtures

		pH
10.00 ml glycine		6.10 <sub>6</sub>
9.90	- 0.10 ccm NaOH	7.80
9.75	- 0.25	8.23 <sub>7</sub>
9.50	- 0.50	8.57
9.00	- 1.00	8.92 <sub>1</sub>
8.00	- 2.00	9.30 <sub>1</sub>
7.00	- 3.00	9.71 <sub>1</sub>
6.00	- 4.00	10.11
5.50	- 4.50	10.48 <sub>7</sub>
5.10	- 4.90	11.06 <sub>7</sub>
5.00	- 5.00	11.30
4.90	- 5.10	11.56
4.50	- 5.50	12.09 <sub>1</sub>
4.00	- 6.00	12.39 <sub>1</sub>
3.00	- 7.00	12.67 <sub>1</sub>
2.00	- 8.00	12.85 <sub>1</sub>
1.00	- 9.00	12.97 <sub>1</sub>
	- 10.00	13.00 <sub>1</sub>



TABLE 28 Borate Mixtures

			pH
10.00 ml borate			9.24 <sub>1</sub>
9.50	+ 0.50 com HCl		9.16 <sub>2</sub>
9.00	+ 1.00		9.06 <sub>7</sub>
8.50	+ 1.50		9.00 <sub>7</sub>
8.00	+ 2.00		8.90 <sub>2</sub>
7.50	+ 2.50	"	8.79 <sub>2</sub>
7.00	+ 3.00	'	8.67 <sub>2</sub>
6.50	+ 3.50		8.50 <sub>2</sub>
6.00	+ 4.00		8.28 <sub>2</sub>
5.75	+ 4.25	"	8.13 <sub>7</sub>
5.50	+ 4.50	'	7.93 <sub>2</sub>
5.25	+ 4.75		7.26 <sub>1</sub>
5.00	+ 5.00		6.54 <sub>2</sub>
4.75	+ 5.25		2.37 <sub>1</sub>

			pH
10.00 ml borate			9.24 <sub>1</sub>
9.00	+ 1 com NaOH		9.36 <sub>2</sub>
8.00	+ 2		9.50 <sub>2</sub>
7.00	+ 3	'	9.67 <sub>2</sub>
6.00	+ 4	'	9.97 <sub>2</sub>
5.00	+ 5		11.07 <sub>2</sub>
4.00	+ 6		12.37 <sub>2</sub>

TABLE 29 Phosphate Mixtures

			pH
10.00 ml sec Phosph			9.18 <sub>2</sub>
9.90	- 0.10 ml prim. Phosph		8.67 <sub>2</sub>
9.75	- 0.25		8.33 <sub>2</sub>
9.50	- 0.50		8.04 <sub>2</sub>
9.00	- 1.00		7.73 <sub>1</sub>
8.00	- 2.00	'	7.38 <sub>2</sub>
7.00	- 3.00		7.16 <sub>2</sub>
6.00	- 4.00		6.97 <sub>2</sub>
5.00	- 5.00		6.87 <sub>2</sub>
4.00	- 6.00		6.64
3.00	- 7.00		6.46 <sub>2</sub>
2.00	- 8.00		6.23
1.00	- 9.00		5.90 <sub>2</sub>
0.50	- 9.50		5.58
0.25	- 9.75	'	5.28 <sub>2</sub>
0.10	- 9.90	'	4.94 <sub>2</sub>

TABLE 30 Acid Citrate Mixtures

		pH
10.00 ml citrate		4.95 <sub>1</sub>
9.50	— 0.50 ml HCl	4.88 <sub>1</sub>
9.00	— 1.00	4.83 <sub>1</sub>
8.00	— 2.00	4.65 <sub>1</sub>
7.00	— 3.00	4.44 <sub>1</sub>
6.00	— 4.00	4.15 <sub>1</sub>
5.50	— 4.50	3.94 <sub>1</sub>
5.00	— 5.00	3.66 <sub>1</sub>
4.75	— 5.25	3.52 <sub>1</sub>
4.50	— 5.50	3.35 <sub>1</sub>
4.00	— 6.00	2.97 <sub>1</sub>
3.83	— 6.67	2.77 <sub>1</sub>
3.00	— 7.00	1.97 <sub>1</sub>
2.00	— 8.00	1.41 <sub>1</sub>
1.00	— 9.00	1.17 <sub>1</sub>
	— 10.00	1.03 <sub>1</sub>

TABLE 31 Alkaline Citrate Mixtures

		pH
10.00 ml citrate		4.95 <sub>1</sub>
9.50	— 0.50 ml NaOH	5.02 <sub>1</sub>
9.00	— 1.00	5.10 <sub>1</sub>
8.00	— 2.00	5.31 <sub>1</sub>
7.00	— 3.00	5.56 <sub>1</sub>
6.00	— 4.00	5.96 <sub>1</sub>
5.50	— 4.50	6.33 <sub>1</sub>
5.25	— 4.75	6.67 <sub>1</sub>
5.00	— 5.00	9.05 <sub>2</sub> (up to)
		10.09 <sub>2</sub>
4.50	— 5.50	12.07 <sub>1</sub>
4.00	— 6.00	12.36

(6) 0.1 molar solution of secondary sodium citrate 21.01 Gm. of citric acid is dissolved in 200 ml. of N/1 NaOH and made up to 1 liter with water

(7) 0.2 molar boric acid 12.40 Gm. of boric acid is dissolved in 100 ml. of N/1 NaOH and made up to 1 liter with water

The distilled water used for these solutions must be freed from  $\text{CO}_2$  by boiling, and the solutions should be kept in bottles, equipped with soda-lime tubes

#### APPARATUS FOR THE PREPARATION OF REDISTILLED WATER<sup>7</sup>

The apparatus consists of 2 distilling vessels (A) and (B), fitting into each other. A is heated by gas or electricity, B by steam created by A. The outer flask A is connected with a constant level arrangement N (fig 71), which allows a continuous water supply, thus replacing the water evaporating in A. When the outer flask is heated the steam gets into the inner distilling vessel B through tube R and from there via 1 and 2 into the condenser K. By condensation in the condenser, part of the water runs down as singly distilled water. Another part, after condensation in tube 1, runs back into vessel B and is condensed there, whereby the lower part of B up to the height of the drainage tube U is constantly filled with water. Continuous heating by steam entering through R and also by the steam in the flask A effects a second distillation. Tube U serves as valve by draining off the condensing water which is not redistilled. Thus, constant pressure and even boiling is maintained. The inner vessel B is connected with A by a ground glass joint and held in place with wire springs. A Frederick condenser ( $K_1$   $K_2$ ) is sealed on to the joint. The apparatus is easy to handle and may be attached to an iron ring stand with heavy clamps.

When the apparatus is adjusted correctly, 1500 ml. of water may be distilled during 1 hour. No supervision is necessary, once the stopcock is opened and the water flow regulated. The specific conduction of the water, thus obtained is  $\kappa = 1.5 \times 10^{-4}$ .

If this apparatus is not available, water may be distilled in the following manner (fig 72)

Essentially the siphon principle is combined with that of the Mariotte bottle. The bottom of the tube B, in the Mariotte bottle A, is adjusted so as to lie in the same horizontal plane as the surface of the water in the flask, C, when the flask has been filled to a level convenient for distillation. In practice the fluid usually rises somewhat higher in the flask C than the level calculated because the air in the Mariotte bottle A becomes progressively hotter as the distillation proceeds due to the small amount of steam which bubbles back

through the tube D. Accordingly, the Mariotte bottle is usual adjusted so that the end of the tube B is in the same horizontal plane as the surface of the liquid in the flask C, when the flask is about two thirds full. The end of the siphon tube, D, is bent slightly upward and outward so that the orifice is near the wall of the flask. The

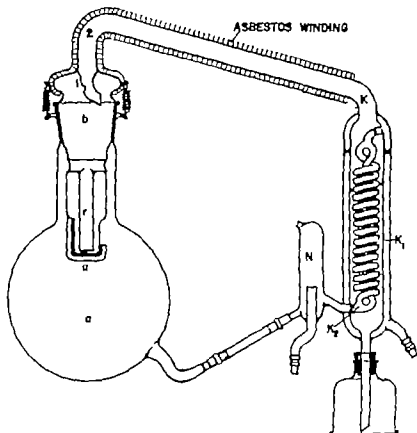


FIG 71 Apparatus for water-distillation with automatic regulation of the liquid level

tends to minimize the amount of steam which always bubbles back up through the tube, D into the Mariotte bottle. Although the steam heats the water in the Mariotte bottles as the distillation progresses, in our experience this has neither interfered with the smooth operation of the unit nor cracked the nonpyrex Mariotte bottle. The lower opening of the tube D must of course be below the lower end of the tube B or the water will fail to siphon into flask

C as the distillation boils off the fluid in flask, C H is merely a plug of glass wool to prevent the entrainment of impure water droplets in the steam which passes over into the condenser, E The area of the condenser surface, if too small will, of course, limit the rate of distillation since some of the steam, when too small a condenser is employed, will pass over into the receiver uncondensed In actual operation the distillation is started with water in flask C, at the usual

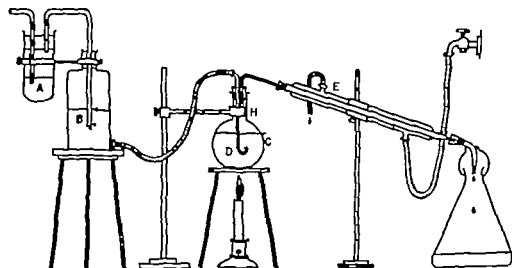


FIG 72 AUTOMATIC DISTILLING UNIT

level, about two-thirds full As the water in C boils down to a critical level about one inch above the bottom of the flask water begins to siphon over into the still, C, from the Mariotte bottle, A. This cool water stops the boiling until flask C is again about two-thirds full, at which point siphoning ceases With each succeeding filling the water will rise to a slightly higher level in flask C, because the air in the Mariotte bottle, as already noted grows hotter as the distillation continues All connections must be airtight and this precaution applies especially to the stopper in the top of the Mariotte bottle With the precautions mentioned the distillation can be started early in the day and will smoothly proceed unattended as long as there is water in the Mariotte bottle

- (I) Conversion of mg. per 100 ml. into millimols per liter (mM.) and vice versa

$$(a) \text{ mM.} = \frac{10 \times \text{mg. per 100 ml.}}{\text{molecular weight}}$$

*Example* 630 mg. per 100 ml. NaCl

$$\frac{10 \times 630}{58.5} = 1077 \text{ mM NaCl}$$

$$(b) \text{ mg. per 100 ml.} = \frac{\text{mM} \times \text{molecular weight}}{10}$$

*Example.* 1077 mM. NaCl

$$\frac{1077 \times 58.5}{10} = 630 \text{ mg. per 100 ml. NaCl}$$

(II) Conversion of mg. per 100 ml. into milli-equivalents per liter (m. Eq.) and vice versa

$$(c) \text{ m. Eq} = \frac{10 \times \text{mg. per 100 ml.} \times \text{valency}}{\text{molecular weight}}$$

*Example.* 3.5 mg. per 100 ml. Mg

$$\frac{10 \times 3.5 \times 2}{24.32} = 2.88 \text{ m. Eq Mg}$$

$$(d) \text{ mg. per 100 ml.} = \frac{\text{m. Eq} \times \text{molecular weight}}{10 \times \text{valency}}$$

*Example.* 2.88 m. Eq Mg

$$\frac{2.88 \times 24.32}{10 \times 2} = 3.5 \text{ mg. per 100 ml. Mg}$$

(III) Conversion of millimols per liter (mM.) into milli-equivalents per liter (m. Eq.) and vice versa

$$(e) \text{ mM.} = \frac{\text{m. Eq}}{\text{valency}}$$

$$(f) \text{ m. Eq} = \text{mM.} \times \text{valency}$$

(IV) Conversion of ml. of gas (at S.T.P.) per 100 ml. into millimols per liter and vice versa.

$$(g) \text{ mM.} = \frac{\text{ml. per 100 ml.}}{2.24}$$

*Example* 65.3 ml. per 100 ml. CO<sub>2</sub>

$$\frac{65.3}{2.24} = 29.15 \text{ mM. CO}_2$$

(h) ml. per 100 ml. = 2.24 × mM.

*Example* 29.1 mM. CO<sub>2</sub>

$$2.24 \times 29.1 = 65.3 \text{ ml. per 100 ml. CO}_2$$

TABLE 32

Conversion of Celsius degrees into Fahrenheit degrees and vice versa.  
Formula (Celsius degrees × 9/5) + 32 = Fahrenheit degrees (Fahrenheit degrees - 32) × 5/9 = Celsius degrees.

C	F	C	F	F	C	F	C
300	572.0	25	77.0	212	100	101	38.3
200	392.0	24	75.2	210	98.9	100	37.8
150	302.0	23	73.4	200	93.8	99	37.2
140	284.0	22	71.6	190	87.7	98	36.7
130	266.0	21	69.8	180	82.2	97	36.1
120	248.0	20	68.0	170	76.6	96	35.6
110	230.0	19	66.2	160	71.1	95	35.0
100	212.0	18	64.4	150	65.6	90	32.2
90	194.0	17	62.6	140	60.0	80	26.7
80	176.0	16	60.8	130	54.4	70	21.1
70	158.0	15	59.0	120	48.9	60	15.6
60	140.0	10	50.0	110	43.8	50	10.0
50	122.0	0	32.0	109	42.7	40	4.4
40	104.0			108	42.2	32	0
39	102.2			107	41.7		
38	100.4			106	41.1		
37	98.6			105	40.6		
36	96.8			104	40.0		
35	95.0			103	39.4		
30	86.0			102	38.9		
29	84.2						
28	82.4						
27	80.6						
26	78.8						

#### PHOTOELECTRIC COLORIMETRY

*Principles* The photoelectric colorimeter is based on the property of light to create an electric potential in a photoelectric cell. The cur

rent output of the cell depends on the intensity of light reaching it. Colored and turbid solutions interposed between the source of light and the cell absorb a part of the light proportional to the concentration of the colored substance or to the degree of turbidity. The photoelectric cell responds with either a change in current passing through it or generating current proportional to the intensity of light falling on it. This current is measured by means of an electrical circuit including a resistance and a galvanometer. The scale of the galvanometer is divided into arbitrary units.

The photoelectric colorimeter may be used for any colorimetric procedure which has been devised for the visual colorimeter. Turbidimetric measurements may be made in the colorimeter just as readily as colorimetric ones; the basis of calibration is then a solution of standard turbidity. The chief advantages of a photoelectric colorimeter are

- (a) elimination of personal error
- (b) greater speed in making readings
- (c) greater accuracy
- (d) elimination of the influence of colored reagents (blank may be substituted for water)

There are two general types of photoelectric colorimeters

(1) The single cell type (Evelyn Coleman) where a barrier layer type of cell is used, the light source being of low intensity and voltage (storage battery or transformer with a voltage stabilizer)

(2) The twin-cell type (Klett-Summerson) equipped with two matched photocells connected in opposition to a galvanometer and mounted one on either side of a central light source. Any variations in illumination due to fluctuation of current affect the two cells equally, so that the fluctuations are eliminated. This type of colorimeter may be attached to any convenient source of alternating or direct current. In both types calibrated test tubes are used for the colored standard solutions.

### *General Information About the Colorimeter\**

1 *Light filters* For the best results in the photoelectric colorimeter it is important that the proper light filter be used. Ordinarily the filter selected is one whose spectral transmission is complementary to that

The information given in this chapter is mainly based on experience with the Klett-Summerson type of colorimeter





acid and their turbidities determined in the colorimeter using a blue filter. Simultaneously the protein nitrogen of each sample is determined by the micro-Kjeldahl method (see p. 218). The mean of the turbidity readings is set against the mean of the protein content as calculated from the Kjeldahl analysis and compared to colorimetric reading of the cobalt standard solution. The correlation factor ( $x$ ) is calculated as follows:

correlation factor ( $x$ )

$$= \frac{\text{mean protein content of CSF (A)} \times \text{cobalt standard reading (C)}}{\text{mean reading of CSF (B)}}$$

The correlation factor gives the number indicating the concentration of protein in CSF (mg. per cent) corresponding to the standard cobalt solution reading.

*Example*

mg. per cent protein acc. to Kjeldahl method (A)	colorimetric reading of protein turbidity (B)	colorimetric reading of cobalt stand (C)
18.6 mg. per cent	42	143
23.8	53	113
35.8	81	143
45.7	106	143
61.3	147	143
83.5	190	113
268.7 mg. per cent	619	113

$$x = \frac{A \times C}{B} = \frac{268.7 \times 143}{619} = 62.08 \text{ mg. per cent protein}$$

This calculation indicates that light absorption of the cobalt standard corresponds to 62.08 mg. per cent (or practically 62.0 mg. per cent) protein. If due to instability of the colorimeter the cobalt absorption value would be some day e.g. 147 instead of 113, the former would correspond to a concentration of 62 mg. per cent protein and all further calculations should be based on this ratio.

*Procedure*

One milliliter of clear, colorless unknown CSF is placed in a colorimeter tube and 0.0 ml. of 3 per cent sulfo-salicylic acid are added.

The contents of the tube are gently mixed. Reading in the colorimeter is made after 10–30 minutes against the blank, containing 1 ml of water or normal saline and 6 ml of 3 per cent sulfosalicylic acid. Before reading, the contents of the tube are again gently mixed.

If the sample shows a strong Pandy's reaction, the amount of fluid used for the determination should be reduced to 0.5 ml or 0.25 ml, adding normal saline to make up to 1 ml, and the calculation adjusted accordingly. If less than 1 ml of fluid is available, the determination can be performed with 0.3 ml. In this case the amount of sulfosalicylic acid is reduced proportionally to 1.8 ml and the reading is made in a micro-tube.

### *Calculation*

Protein content of unknown

$$= \frac{\text{reading of unknown} \times \text{correlation factor}}{\text{reading of cobalt standard}}$$

### *Example*

If cobalt standard solution reading is today—147  
and it corresponds to a concentration of —62 mg per cent protein (correlation factor)  
and the reading of the unknown is—88  
then the concentration of the unknown is

$$\frac{88 \times 62}{147} = 37 \text{ mg. per cent}$$

### *Remarks*

This method is not suitable for the estimation of total proteins in serum, as under certain pathologic conditions protein compounds of varying molecular weight appear and cause varying turbidities when the fluid is treated with sulfosalicylic acid.

## DETERMINATION OF SERUM IRON\* (p 129)

### *Principle*

Phenantroline reacts with ferrous iron in serum, producing a red color, the intensity of which is determined in the colorimeter.

\* Klett Summerson Clinical Manual

*Reagents*

(1) 0.3 N HCl 30 ml of concentrated HCl are diluted to 1 liter with distilled water

(2) 20 per cent trichloroacetic acid The purest grades of trichloroacetic acid contain traces of iron and should be purified by vacuum distillation

(3) 50 per cent ammonium acetate

(4) 1 per cent hydroquinone 1 Gm of pure hydroquinone is dissolved in 100 ml of water, acidified with one drop of concentrated  $\text{H}_2\text{SO}_4$ . This solution may be preserved for weeks in a dark bottle and on ice but it must be discarded when a brown color appears

(5) 0.1 per cent p-phenantroline monohydrate solution in water This reagent should be discarded when any color appears

(6) iron standard solution

(a) stock solution 1.4016 Gm of ferro-ammonium sulfate and a few ml of HCl (1) are dissolved in 1 liter of water and acidified with 1 ml of  $\text{H}_2\text{SO}_4$

(b) working solution 2 ml of stock solution (6a) plus a few drops of HCl are diluted to 100 ml with water This solution contains 2  $\gamma$  of iron per ml and is very stable

*Procedure*

One ml of 0.3 N HCl (1) is added to 2 ml of serum After 1 hour 1 ml of 20 per cent trichloroacetic acid solution (2) is added left standing for about 10 minutes, centrifuged and filtered through an iron free filter (Whatman 42) Two milliliters of this filtrate are transferred to a colorimeter tube and the following solutions are added 0.1 ml of 50 per cent ammonium acetate solution (3) 0.15 ml of 1 per cent hydroquinone solution (4), and 0.5 ml of 0.1 per cent p-phenantroline monohydrate solution (5) When the pink color has fully developed (after about 20 minutes) the reading is made in the colorimeter against the blank tube set at 0 The color is stable

*Standard*

Two ml of standard iron solution (6) are mixed with 1 ml of 0.3 N HCl (1) and 1 ml of 20 per cent trichloroacetic acid Two ml of the mixture are placed into a colorimeter tube ammonium acetate,

hydroquinone, and p-phenantrolme are added exactly as described above. The reading is performed against the blank tube set at following the time conditions specified for the unknown.

### Blank

Two ml. of distilled water are used instead of serum or standard and treated exactly as above.

### Calculation

against the standard

$$\frac{200}{\text{reading of standard}} \times \text{reading of unknown} = \text{micrograms per cent iron in the serum}$$

The standard is the equivalent of a serum containing 200  $\gamma$  per cent iron. The proportionality holds very well for all values up to 1000.

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- Thymol bromide, 210  
Total proteins (blood), 218  
    (CSF) 231  
Tributyrin 339  
Tropoline solution 330  
  
Urease suspension 153 157  
    from Jack Bean meal 160 162  
Uric acid reagent, 193  
  
Van Slyke apparatus (manometer) 36  
    (vol) 27  
  
Veratrol 239  
Vitamin C 342  
    K 347 350  
  
Widmark alcohol flask 236  
  
Xanthochrome reaction 325  
Xanthoprotein reaction, 214  
  
Yeast, growth 266  
    suspension 265  
  
Zinc sulfate potassium iodide reagent 250

